

5-FU⁴ is widely used in the treatment of a range of cancers including colorectal, breast and cancers of the aerodigestive tract. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) (Longley et al., 2003). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH₂THF) as the methyl donor. This reaction provides the sole intracellular

1 source of thymidylate, which is essential for DNA
2 synthesis and repair. The 5-FU metabolite
3 fluorodeoxyuridine monophosphate (FdUMP) forms a
4 stable complex with TS and CH₂THF resulting in enzyme
5 inhibition (Longley et al., 2003). Recently, more
6 specific folate-based inhibitors of TS have been
7 developed such as tomudex (TDX) and Alimta (MTA),
8 which form a stable complex with TS and dUMP that
9 inhibits binding of CH₂THF to the enzyme (Hughes et
10 al., 1999; Shih et al., 1997). TS inhibition causes
11 nucleotide pool imbalances that result in S phase cell
12 cycle arrest and apoptosis (Aherne et al., 1996;
13 Longley et al., 2002; Longley et al., 2001).
14 Oxaliplatin is a third generation platinum-based DNA
15 damaging agent that is used in combination with 5-FU
16 in the treatment of advanced colorectal cancer
17 (Giacchetti et al., 2000). Drug resistance is a major
18 factor limiting the effectiveness of chemotherapies.
19 The topoisomerase-1 inhibitor irinotecan (CPT-11) and
20 the DNA damaging agent oxaliplatin are now being used
21 in conjunction with 5-FU for the treatment of
22 metastatic colorectal cancer, having demonstrated
23 improved response rates compared to treatment with 5-
24 FU alone (40-50% compared to 10-15%) (10, 11). Despite
25 these improvements, the vast majority of responding
26 patients relapse, with median survival times of only
27 22-24 months. Clearly, new approaches are needed for
28 the treatment of this disease.

29

30 Death receptors such as Fas and the TRAIL (tumour
31 necrosis factor (TNF)-related apoptosis-inducing
32 ligand) receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2)

1 trigger death signals when bound by their natural
2 ligands (1,2). Ligand binding to the death receptors
3 leads to recruitment of the adaptor protein FADD
4 (Fas-associated death domain), which in turn
5 recruits procaspase 8 zymogens to form the death-
6 inducing signalling complex (DISC) (Nagata, 1999).
7 Procaspase 8 molecules become activated at the DISC
8 and subsequently activate pro-apoptotic downstream
9 molecules such as caspase 3 and BID. FasL expression
10 is up-regulated in most colon tumours, and it has
11 been postulated that tumour FasL induces apoptosis
12 of Fas-sensitive immune effector cells (O'Connell et
13 al., 1999). This mechanism of immune escape requires
14 that tumour cells develop resistance to Fas-mediated
15 apoptosis to prevent autocrine and paracrine tumour
16 cell death.

17
18 A key inhibitor of Fas signaling is c-FLIP, which
19 inhibits procaspase 8 recruitment and processing at
20 the DISC (Krueger et al., 2001). Differential
21 splicing gives rise to long (c-FLIP_L) and short (c-
22 FLIP_S) forms of c-FLIP, both of which bind to FADD
23 within the DISC. c-FLIP_S directly inhibits caspase 8
24 activation at the DISC, whereas c-FLIP_L is first
25 cleaved to a p43 truncated form that inhibits
26 complete processing of procaspase 8 to its active
27 subunits. c-FLIP also inhibits procaspase 8
28 activation at DISCs formed by the TRAIL (TNF-related
29 apoptosis-inducing ligand) death receptors DR4
30 (TRAIL-R1) and DR5 (TRAIL-R2) (Krueger et al.,
31 2001). In addition to blocking caspase 8 activation,
32 DISC-bound c-FLIP has been reported to promote

1 activation of the ERK, PI3-kinase/Akt and NF- κ B
2 signaling pathways (Krueger et al., 2001). Thus, c-
3 FLIP potentially converts death receptor signaling
4 from pro- to anti-apoptotic by activating intrinsic
5 survival pathways. Significantly, c-FLIP_L has been
6 found to be overexpressed in colonic adenocarcinomas
7 compared to matched normal tissue, suggesting that
8 c-FLIP may contribute to in vivo tumour
9 transformation (Ryu et al., 2001).

10

11 **Summary of the Invention**

12

13 As described herein and, as shown in our co-pending
14 PCT application filed on the same day as the present
15 application and claiming priority from GB patent
16 application 0327493.3, the present inventors have
17 shown that by combining treatment using a death
18 receptor ligand, such as an anti FAS antibody, for
19 example, CH-11, with a chemotherapeutic agent such
20 as 5-FU or an antifolate drug, such as raltitrexed
21 (RTX) or pemetrexed (MTA, Alimta), a synergistic
22 effect is achieved in the killing of cancer cells.
23 However, the synergistic effect achieved was
24 abrogated in cancer cells which overexpress c-FLIP.

25

26 As described in the Examples, in cell lines which
27 demonstrated overexpression of c-FLIP and associated
28 resistance to chemotherapy e.g 5-FU induced
29 apoptosis, inhibition of FLIP expression reversed
30 the resistance to chemotherapy-induced apoptosis.
31 On further investigating this effect, the inventors

1 tested a number of cell lines having a p53 mutation
2 or p53 null genotype.

3
4 To their surprise, the inventors observed that down-
5 regulation of c-FLIP markedly enhanced apoptosis in
6 response to certain chemotherapeutic agents in the
7 p53 mutant cells, which are usually highly resistant
8 to the particular chemotherapeutic agents. This
9 surprising observation enables the use of
10 combinations of such cFLIP inhibitors and
11 chemotherapeutic agents in the treatment of cancers
12 associated with p53 mutations.

13
14 Accordingly, in a first aspect of the present
15 invention, there is provided a method of killing
16 cancer cells having a p53 mutation, comprising
17 administration to said cells of:

- 18 (a) a c-FLIP inhibitor and
19 (b) a chemotherapeutic agent, wherein the
20 chemotherapeutic agent is a thymidylate synthase
21 inhibitor, a platinum cytotoxic agent or a
22 topoisomerase inhibitor.

23
24 In a second aspect, there is provided a method of
25 treating cancer associated with a p53 mutation
26 comprising administration to a subject in need
27 thereof of

- 28 (a) a c-FLIP inhibitor and
29 (b) a chemotherapeutic agent, wherein the
30 chemotherapeutic agent is a thymidylate synthase
31 inhibitor, a platinum cytotoxic agent or a

1 topoisomerase inhibitor.

2

3 A third aspect of the invention comprises the use of

4 (a) a c-FLIP inhibitor and

5 (b) a chemotherapeutic agent, wherein the

6 chemotherapeutic agent is a thymidylate synthase

7 inhibitor, a platinum cytotoxic agent or a

8 topoisomerase inhibitor

9 in the preparation of a medicament for treating

10 cancer associated with a p53 mutation.

11

12 A fourth aspect provides a pharmaceutical

13 composition for the treatment of a cancer associated

14 with a p53 mutation, wherein the composition

15 comprises (a) a c-FLIP inhibitor

16 (b) a chemotherapeutic agent, wherein the

17 chemotherapeutic agent is a thymidylate synthase

18 inhibitor, a platinum cytotoxic agent or a

19 topoisomerase inhibitor

20 and

21 (c) a pharmaceutically acceptable excipient, diluent

22 or carrier.

23

24 A fifth aspect provides a kit for the treatment of

25 cancer associated with a p53 mutation, said kit

26 comprising

27 (a) a c-FLIP inhibitor and

28 (b) a chemotherapeutic agent, wherein the

29 chemotherapeutic agent is a thymidylate synthase

30 inhibitor, a platinum cytotoxic agent or a

31 topoisomerase inhibitor and

32 (c) instructions for the administration of (a) and

1 (b) separately, sequentially or simultaneously.

2

3 In any of the first to fifth aspects of the
4 invention, the c-FLIP inhibitor and the
5 chemotherapeutic agent may be provided and
6 administered in the absence of other active agents.
7 However, in a preferred embodiment of these aspects
8 aspects of the invention, there is provided (c) a
9 death receptor binding member, or a nucleic acid
10 encoding said binding member.

11

12 Any suitable death receptor binding member may be
13 used. Death receptors include, Fas, TNFR, DR-3, DR-4
14 and DR-5. In preferred embodiments of the invention,
15 the death receptor is FAS.

16

17 The c-FLIP inhibitor, the chemotherapeutic agent
18 and where applicable the death receptor ligand, may
19 be administered simultaneously, sequentially or
20 simultaneously. In preferred embodiments of the
21 invention, the c-FLIP inhibitor is administered
22 prior to the chemotherapeutic agent and, where
23 applicable, the specific binding member.

24

25 A preferred binding member for use in the invention
26 is an antibody or a fragment thereof. In
27 particularly preferred embodiments, the binding
28 member is the FAS antibody CH11 (Yonehara, S.,
29 Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169,
30 1747-1756) (available commercially e.g. from Upstate
31 Biotechnology, Lake Placid, NY).

32

1 Any suitable thymidylate synthase inhibitor,
2 platinum cytotoxic agent or topoisomerase inhibitor
3 may be used in the present invention. Examples of
4 thymidylate synthase inhibitors which may be used in
5 the methods of the invention include 5-FU, MTA and
6 TDX. In a preferred embodiment, the thymidylate
7 synthase inhibitor is 5-FU. Examples of platinum
8 cytotoxic agents which may be used include cisplatin
9 and oxaliplatin. In a particularly preferred
10 embodiment of the invention, the chemotherapeutic
11 agent is cisplatin. Any suitable topoisomerase
12 inhibitor may be used in the present invention. In
13 a preferred embodiment, the topoisomerase inhibitor
14 is a topoisomerase I inhibitor, for example a
15 camptothecin. A suitable topoisomerase I inhibitor,
16 which may be used in the present invention is
17 irinotecan (CPT-11). Unless, the context demand
18 otherwise, reference to CPT-11 should be taken to
19 encompass CPT-11 or its active metabolite SN-38.

20
21 In preferred embodiments of the invention, the c-
22 FLIP inhibitor and the chemotherapeutic agent are
23 administered in a potentiating ratio. the term
24 "potentiating ratio" in the context of the present
25 invention is used to indicate that the cFLIP
26 inhibitor and chemotherapeutic agent are present in
27 a ratio such that the cytotoxic activity of the
28 combination is greater than that of either component
29 alone or of the additive activity that would be
30 predicted for the combinations based on the
31 activities of the individual components. Thus in a

1 potentiating ratio, the individual components act
2 synergistically.

3

4 Synergism may be defined using a number of methods.
5 For example, synergism may be defined as an RI of
6 greater than unity using the method of Kern as
7 modified by Romaneli (1998a, 1998b). The RI may be
8 calculated as the ratio of expected cell survival
9 (S_{exp} , defined as the product of the survival
10 observed with drug A alone and the survival observed
11 with drug B alone) to the observed cell survival
12 (S_{obs}) for the combination of A and B ($RI = S_{exp}/S_{obs}$).
13 Synergism may then be defined as an RI of greater
14 than unity.

15

16 In another method, synergism may be determined by
17 calculating the combination index (CI) according to
18 the method of Chou and Talalay. CI values of 1, <1,
19 and >1 indicate additive, synergistic and
20 antagonistic effects respectively.

21

22 In a preferred embodiment of the invention, the c-
23 FLIP inhibitor and the chemotherapeutic agent are
24 present in concentrations sufficient to produce a CI
25 of less than 1, preferably less than 0.85.

26

27 Synergism is preferably defined as an RI of greater
28 than unity using the method of Kern as modified by
29 Romaneli (1998a,b)). The RI may be calculated as the
30 ratio of expected cell survival (S_{exp} , defined as the
31 product of the survival observed with drug A alone
32 and the survival observed with drug B alone) to the

1 observed cell survival (S_{obs}) for the combination of
2 A and B ($RI = S_{exp} / S_{obs}$). Synergism may then be defined
3 as an RI of greater than unity.

4
5 In preferred embodiments of the invention, said
6 specific binding member and chemotherapeutic agent
7 are provided in concentrations sufficient to produce
8 an RI of greater than 1.5, more preferably greater
9 than 2.0, most preferably greater than 2.25.

10
11 The combined medicament thus preferably produces a
12 synergistic effect when used to treat tumour cells.

13
14 The invention according to any of the first, second
15 third, fourth and fifth aspect of the invention may
16 be used for the killing of any cancer cell having a
17 p53 mutation. The mutation may partially or totally
18 inactivate p53 in a cell. In one embodiment of the
19 invention, the p53 mutation is a p53 mutation, which
20 totally inactivates p53. In another embodiment, the
21 p53 mutation is a missense mutation resulting in the
22 substitution of histidine (R175H mutation). In
23 another embodiment, the p53 mutation is a missense
24 mutation resulting in the substitution of tryptophan
25 (R248W mutation) for arginine.

26
27 As described in the Examples, as well as testing the
28 cytotoxicity of combinations of c-FLIP inhibitors
29 and chemotherapeutic agents on cancer cells, the
30 inventors further tested the effects of c-FLIP
31 alone. The inventors unexpectedly observed that
32 relatively potent inhibition of cFLIP using high

1 concentrations of siRNA triggered apoptosis in the
2 absence of chemotherapy in both RKO and H630 cell
3 lines. This demonstration that cFLIP inhibition in
4 the absence of chemotherapy is sufficient to trigger
5 apoptosis in cancer cells enables the use of c-FLIP
6 inhibition alone as a chemotherapeutic strategy.

7
8 Accordingly, in a sixth aspect of the invention,
9 there is provided a method of killing cancer cells,
10 comprising administration to said cells of an
11 effective amount of a c-FLIP inhibitor, wherein the
12 c-FLIP inhibitor is administered as the sole
13 cytotoxic agent in the substantial absence of other
14 cytotoxic agents.

15
16 A seventh aspect of the invention provides a method
17 of treating cancer comprising administration to a
18 subject in need thereof a therapeutically effective
19 amount of a c-FLIP inhibitor, wherein the c-FLIP
20 inhibitor is administered as the sole cytotoxic
21 agent in the substantial absence of other cytotoxic
22 agents.

23
24 An eighth aspect provides the use of a c-FLIP
25 inhibitor as the sole cytotoxic agent in the
26 preparation of a medicament for treating cancer,
27 wherein the medicament is for treatment in the
28 substantial absence of other cytotoxic agents.

29
30 A ninth aspect provides a pharmaceutical composition
31 for the treatment of cancer, wherein the composition
32 comprises a c-FLIP inhibitor as the sole cytotoxic

1 agent and a pharmaceutically acceptable excipient,
2 diluent or carrier, wherein the composition is for
3 treatment in the absence of other cytotoxic agents.

4
5 The sixth to ninth aspects of the invention may be
6 used in the treatment of any cancer. The cancer
7 cells may comprise a p53 wild type genotype or,
8 alternatively, may comprise p53 mutant genotypes.
9 The mutation may partially or totally inactivate p53
10 in a cell. In one embodiment of the invention, the
11 p53 mutation is a p53 mutation, which totally
12 inactivates p53. In another embodiment, the p53
13 mutation is a missense mutation resulting in the
14 substitution of histidine (R175H mutation). In
15 another embodiment, the p53 mutation is a missense
16 mutation resulting in the substitution of tryptophan
17 (R248W mutation) for arginine.

18
19 Any suitable c-FLIP inhibitor may be used in methods
20 of the invention. The inhibitor may be peptide or
21 non-peptide.

22
23 In one preferred embodiment, said c-FLIP inhibitor
24 is an antisense molecule which modulates the
25 expression of the gene encoding c-FLIP.

26
27 In a more preferred embodiment, said c-FLIP
28 inhibitor is an RNAi agent, which modulates
29 expression of the c-FLIP gene. The agent may be an
30 siRNA, an shRNA, a ddRNAi construct or a
31 transcription template thereof, e.g., a DNA encoding
32 an shRNA. In preferred embodiments the RNAi agent

1 is an siRNA which is homologous to a part of the
2 mRNA sequence of the gene encoding c-FLIP.

3

4 Preferred RNAi agents of and for use in the
5 invention are between 15 and 25 nucleotides in
6 length, preferably between 19 and 22 nucleotides,
7 most preferably 21 nucleotides in length. In
8 particularly preferred embodiments of the invention,
9 the RNAi agent has the nucleotide sequence shown as
10 SEQ ID NO: 1.

11

12 AAG CAG TCT GTT CAA GGA GCA (SEQ ID NO: 1)

13

14 In another particularly preferred embodiment of the
15 invention, the RNAi agent has the nucleotide
16 sequence shown as SEQ ID NO: 2

17

18 AAG GAA CAG CTT GGC GCT CAA (SEQ ID NO: 2)

19

20 Indeed such RNAi agents represents a tenth and
21 eleventh independent aspects of the present
22 invention.

23

24 According to a further aspect of the invention,
25 there is provided a vector comprising the RNAi agent
26 of the tenth aspect of the invention.

27

28 In a further aspect, there is provided a kit for the
29 treatment of cancer associated with a p53 mutation,
30 said kit comprising

31 (a) a c-FLIP inhibitor and

32 (b) a chemotherapeutic agent, wherein the

1 chemotherapeutic agent is a thymidylate synthase
2 inhibitor, a platinum cytotoxic agent or a
3 topoisomerase inhibitor and
4 (c) instructions for the administration of (a) and
5 (b) separately, sequentially or simultaneously.

6

7 Preferred features of each aspect of the invention
8 are as for each of the other aspects mutatis
9 mutandis unless the context demands otherwise.

10

11 **Detailed Description**

12

13 As described above, the present invention relates to
14 methods of treatment of cancer, involving cFLIP
15 inhibition.

16

17 The methods of the invention may involve the
18 determination of expression of FLIP protein.

19

20 The expression of FLIP may be measured using any
21 technique known in the art. Either mRNA or protein
22 can be measured as a means of determining up-or down
23 regulation of expression of a gene. Quantitative
24 techniques are preferred. However semi-quantitative
25 or qualitative techniques can also be used. Suitable
26 techniques for measuring gene products include, but
27 are not limited to, SAGE analysis, DNA microarray
28 analysis, Northern blot,
29 Western blot, immunocytochemical analysis, and
30 ELISA.

31

1 RNA can be detected using any of the known
2 techniques in the art. Preferably an amplification
3 step is used as the amount of RNA from the sample
4 may be very small. Suitable techniques may include
5 real-time RT-PCR, hybridisation of copy mRNA (cRNA)
6 to an array of nucleic acid probes and Northern
7 Blotting.

8
9 For example, when using mRNA detection, the method
10 may be carried out by converting the isolated mRNA
11 to cDNA according to standard methods; treating the
12 converted cDNA with amplification reaction reagents
13 (such as cDNA PCR reaction reagents) in a container
14 along with an appropriate mixture of nucleic acid
15 primers; reacting the contents of the container to
16 produce amplification products; and analyzing the
17 amplification products to detect the presence of
18 gene expression products of one or more of the genes
19 encoding FLIP protein. Analysis may be accomplished
20 using Southern Blot analysis to detect the presence
21 of the gene products in the amplification product.
22 Southern Blot analysis is known in the art. The
23 analysis step may be further accomplished by
24 quantitatively detecting the presence of such gene
25 products in the amplification products, and
26 comparing the quantity of product detected against a
27 panel of expected values for known presence or
28 absence in normal and malignant tissue derived using
29 similar primers.

30
31 In e.g. determining gene expression in carrying out
32 conventional molecular biological, microbiological

1 and recombinant DNA techniques techniques known in
2 the art may be employed. Details of such
3 techniques are described in, for example, Sambrook,
4 Fritsch and Maniatis, "Molecular Cloning, A
5 Laboratory Manual, Cold Spring Harbor Laboratory
6 Press, 1989, and Ausubel et al, Short Protocols in
7 Molecular Biology, John Wiley and Sons, 1992).

8

9 **Binding members**

10

11 In the context of the present invention, a "binding
12 member" is a molecule which has binding specificity
13 for another molecule, in particular a receptor,
14 preferably a death receptor. The binding member may
15 be a member of a pair of specific binding members.
16 The members of a binding pair may be naturally
17 derived or wholly or partially synthetically
18 produced. One member of the pair of molecules may
19 have an area on its surface, which may be a
20 protrusion or a cavity, which specifically binds to
21 and is therefore complementary to a particular
22 spatial and polar organisation of the other member
23 of the pair of molecules. Thus, the members of the
24 pair have the property of binding specifically to
25 each other. A binding member of the invention and
26 for use in the invention may be any moiety, for
27 example an antibody or ligand, which preferably can
28 bind to a death receptor.

29

30 The binding member may bind to any death receptor.
31 Death receptors include, Fas, TNFR, DR-3, DR-4 and

1 DR-5. In preferred embodiments of the invention, the
2 death receptor is FAS.

3

4 In preferred embodiments, the binding member
5 comprises at least one human constant region.

6

7 **Antibodies**

8

9 An "antibody" is an immunoglobulin, whether natural
10 or partly or wholly synthetically produced. The
11 term also covers any polypeptide, protein or peptide
12 having a binding domain which is, or is homologous
13 to, an antibody binding domain. These can be
14 derived from natural sources, or they may be partly
15 or wholly synthetically produced. Examples of
16 antibodies are the immunoglobulin isotypes and their
17 isotypic subclasses and fragments which comprise an
18 antigen binding domain such as Fab, scFv, Fv, dAb,
19 Fd; and diabodies.

20

21 A binding member for use in certain embodiments, the
22 invention may be an antibody such as a monoclonal or
23 polyclonal antibody, or a fragment thereof. The
24 constant region of the antibody may be of any class
25 including, but not limited to, human classes IgG,
26 IgA, IgM, IgD and IgE. The antibody may belong to
27 any sub class e.g. IgG1, IgG2, IgG3 and IgG4. IgG1
28 is preferred.

29

30 As antibodies can be modified in a number of ways,
31 the term "antibody" should be construed as covering
32 any binding member or substance having a binding

1 domain with the required specificity. Thus, this
2 term covers antibody fragments, derivatives,
3 functional equivalents and homologues of antibodies,
4 including any polypeptide comprising an
5 immunoglobulin binding domain, whether natural or
6 wholly or partially synthetic. Chimeric molecules
7 comprising an immunoglobulin binding domain, or
8 equivalent, fused to another polypeptide are
9 therefore included. Cloning and expression of
10 chimeric antibodies are described in EP-A-0120694
11 and EP-A-0125023.

12
13 Examples of such fragments which can be used in the
14 invention include the Fab fragment, the Fd fragment,
15 the Fv fragment, the dAb fragment (Ward, E.S. et
16 al., Nature 341:544-546 (1989)), F(ab')₂ fragments,
17 single chain Fv molecules (scFv), bispecific single
18 chain Fv dimers (PCT/US92/09965) and "diabodies",
19 multivalent or multispecific fragments constructed
20 by gene fusion (WO94/13804; P. Hollinger et al.,
21 Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

22
23 A fragment of an antibody or of a polypeptide for
24 use in the present invention generally means a
25 stretch of amino acid residues of at least 5 to 7
26 contiguous amino acids, often at least about 7 to 9
27 contiguous amino acids, typically at least about 9
28 to 13 contiguous amino acids, more preferably at
29 least about 20 to 30 or more contiguous amino acids
30 and most preferably at least about 30 to 40 or more
31 consecutive amino acids.

32

1 A "derivative" of such an antibody or polypeptide,
2 or of a fragment antibody means an antibody or
3 polypeptide modified by varying the amino acid
4 sequence of the protein, e.g. by manipulation of the
5 nucleic acid encoding the protein or by altering the
6 protein itself. Such derivatives of the natural
7 amino acid sequence may involve insertion, addition,
8 deletion and/or substitution of one or more amino
9 acids, preferably while providing a peptide having
10 death receptor, e.g. FAS neutralisation and/or
11 binding activity. Preferably such derivatives
12 involve the insertion, addition, deletion and/or
13 substitution of 25 or fewer amino acids, more
14 preferably of 15 or fewer, even more preferably of
15 10 or fewer, more preferably still of 4 or fewer and
16 most preferably of 1 or 2 amino acids only.

17

18 In preferred embodiments, the binding member is
19 humanised. Methods for making humanised antibodies
20 are known in the art e.g see U.S. Patent No.
21 5,225,539. A humanised antibody may be a modified
22 antibody having the hypervariable region of a
23 monoclonal antibody and the constant region of a
24 human antibody. Thus the binding member may
25 comprise a human constant region. The variable
26 region other than the hypervariable region may also
27 be derived from the variable region of a human
28 antibody and/or may also be derived from a
29 monoclonal antibody. In such case, the entire
30 variable region may be derived from murine
31 monoclonal antibody and the antibody is said to be
32 chimerised. Methods for making chimerised

1 antibodies are known in the art (e.g see U.S. Patent
2 Nos. 4,816,397 and 4,816,567).

3
4 It is possible to take monoclonal and other
5 antibodies and use techniques of recombinant DNA
6 technology to produce other antibodies or chimeric
7 molecules which retain the specificity of the
8 original antibody. Such techniques may involve
9 introducing DNA encoding the immunoglobulin variable
10 region, or the complementary determining regions
11 (CDRs), of an antibody to the constant regions, or
12 constant regions plus framework regions, of a
13 different immunoglobulin. See, for instance, EP-A-
14 184187, GB 2188638A or EP-A-239400. A hybridoma or
15 other cell producing an antibody may be subject to
16 genetic mutation or other changes, which may or may
17 not alter the binding specificity of antibodies
18 produced.

19
20 A typical antibody for use in the present invention
21 is a humanised equivalent of CH11 or any chimerised
22 equivalent of an antibody that can bind to the FAS
23 receptor and any alternative antibodies directed at
24 the FAS receptor that have been chimerised and can
25 be use in the treatment of humans. Furthermore, the
26 typical antibody is any antibody that can cross-
27 react with the extracellular portion of the FAS
28 receptor and either bind with high affinity to the
29 FAS receptor, be internalised with the FAS receptor
30 or trigger signalling through the FAS receptor.

31

32 **Production of Binding Members**

1
2 Binding members, which may be used in certain
3 aspects of the present invention may be generated
4 wholly or partly by chemical synthesis. The binding
5 members can be readily prepared according to well-
6 established, standard liquid or, preferably, solid-
7 phase peptide synthesis methods, general
8 descriptions of which are broadly available (see,
9 for example, in J.M. Stewart and J.D. Young, Solid
10 Phase Peptide Synthesis, 2nd edition, Pierce
11 Chemical Company, Rockford, Illinois (1984), in M.
12 Bodanzsky and A. Bodanzsky, The Practice of Peptide
13 Synthesis, Springer Verlag, New York (1984); and
14 Applied Biosystems 430A Users Manual, ABI Inc.,
15 Foster City, California), or they may be prepared in
16 solution, by the liquid phase method or by any
17 combination of solid-phase, liquid phase and
18 solution chemistry, e.g. by first completing the
19 respective peptide portion and then, if desired and
20 appropriate, after removal of any protecting groups
21 being present, by introduction of the residue X by
22 reaction of the respective carbonic or sulfonic acid
23 or a reactive derivative thereof.
24
25 Another convenient way of producing a binding member
26 suitable for use in the present invention is to
27 express nucleic acid encoding it, by use of nucleic
28 acid in an expression system. Thus the present
29 invention further provides the use of (a) nucleic
30 acid encoding a specific binding member which binds
31 to a cell death receptor and (b) a chemotherapeutic
32 agent and (c) a CFLIP inhibitor in the preparation

1 of a medicament for treating cancer associated with
2 a p53 mutation.

3
4 Nucleic acids of and/or for use in accordance with
5 the present invention may comprise DNA or RNA and
6 may be wholly or partially synthetic. In a preferred
7 aspect, nucleic acid for use in the invention codes
8 for a binding member of the invention as defined
9 above. The skilled person will be able to determine
10 substitutions, deletions and/or additions to such
11 nucleic acids which will still provide a binding
12 member suitable for use in the present invention.

13
14 Nucleic acid sequences encoding a binding member for
15 use with the present invention can be readily
16 prepared by the skilled person using the information
17 and references contained herein and techniques known
18 in the art (for example, see Sambrook, Fritsch and
19 Maniatis, "Molecular Cloning", A Laboratory Manual,
20 Cold Spring Harbor Laboratory Press, 1989, and
21 Ausubel et al, Short Protocols in Molecular Biology,
22 John Wiley and Sons, 1992), given the nucleic acid
23 sequences and clones available. These techniques
24 include (i) the use of the polymerase chain reaction
25 (PCR) to amplify samples of such nucleic acid, e.g.
26 from genomic sources, (ii) chemical synthesis, or
27 (iii) preparing cDNA sequences. DNA encoding
28 antibody fragments may be generated and used in any
29 suitable way known to those of skill in the art,
30 including by taking encoding DNA, identifying
31 suitable restriction enzyme recognition sites either
32 side of the portion to be expressed, and cutting out

1 said portion from the DNA. The portion may then be
2 operably linked to a suitable promoter in a standard
3 commercially available expression system. Another
4 recombinant approach is to amplify the relevant
5 portion of the DNA with suitable PCR primers.
6 Modifications to the sequences can be made, e.g.
7 using site directed mutagenesis, to lead to the
8 expression of modified peptide or to take account of
9 codon preferences in the host cells used to express
10 the nucleic acid.

11
12 The nucleic acid may be comprised as construct(s) in
13 the form of a plasmid, vector, transcription or
14 expression cassette which comprises at least one
15 nucleic acid as described above. The construct may
16 be comprised within a recombinant host cell which
17 comprises one or more constructs as above.
18 Expression may conveniently be achieved by culturing
19 under appropriate conditions recombinant host cells
20 containing the nucleic acid. Following production
21 by expression a specific binding member may be
22 isolated and/or purified using any suitable
23 technique, then used as appropriate.

24
25 Binding members-encoding nucleic acid molecules and
26 vectors for use in accordance with the present
27 invention may be provided isolated and/or purified,
28 e.g. from their natural environment, in
29 substantially pure or homogeneous form, or, in the
30 case of nucleic acid, free or substantially free of
31 nucleic acid or genes of origin other than the

1 sequence encoding a polypeptide with the required
2 function.

3
4 Systems for cloning and expression of a polypeptide
5 in a variety of different host cells are well known.
6 Suitable host cells include bacteria, mammalian
7 cells, yeast and baculovirus systems. Mammalian
8 cell lines available in the art for expression of a
9 heterologous polypeptide include Chinese hamster
10 ovary cells, HeLa cells, baby hamster kidney cells,
11 NSO mouse melanoma cells and many others. A common,
12 preferred bacterial host is E. coli.

13
14 The expression of antibodies and antibody fragments
15 in prokaryotic cells such as E. coli is well
16 established in the art. For a review, see for
17 example Plückthun, Bio/Technology 9:545-551 (1991).
18 Expression in eukaryotic cells in culture is also
19 available to those skilled in the art as an option
20 for production of a binding member, see for recent
21 review, for example Reff, Curr. Opinion Biotech.
22 4:573-576 (1993); Trill et al., Curr. Opinion
23 Biotech. 6:553-560 (1995).

24
25 Suitable vectors can be chosen or constructed,
26 containing appropriate regulatory sequences,
27 including promoter sequences, terminator sequences,
28 polyadenylation sequences, enhancer sequences,
29 marker genes and other sequences as appropriate.
30 Vectors may be plasmids, viral e.g. 'phage, or
31 phagemid, as appropriate. For further details see,
32 for example, Sambrook et al., Molecular Cloning: A

1 Laboratory Manual: 2nd Edition, Cold Spring Harbor
2 Laboratory Press (1989). Many known techniques and
3 protocols for manipulation of nucleic acid, for
4 example in preparation of nucleic acid constructs,
5 mutagenesis, sequencing, introduction of DNA into
6 cells and gene expression, and analysis of proteins,
7 are described in detail in Ausubel et al. eds.,
8 Short Protocols in Molecular Biology, 2nd Edition,
9 John Wiley & Sons (1992).

10
11 The nucleic acid may be introduced into a host cell
12 by any suitable means. The introduction may employ
13 any available technique. For eukaryotic cells,
14 suitable techniques may include calcium phosphate
15 transfection, DEAE-Dextran, electroporation,
16 liposome-mediated transfection and transduction
17 using retrovirus or other virus, e.g. vaccinia or,
18 for insect cells, baculovirus. For bacterial cells,
19 suitable techniques may include calcium chloride
20 transformation, electroporation and transfection
21 using bacteriophage.

22
23 Marker genes such as antibiotic resistance or
24 sensitivity genes may be used in identifying clones
25 containing nucleic acid of interest, as is well
26 known in the art.

27
28 The introduction may be followed by causing or
29 allowing expression from the nucleic acid, e.g. by
30 culturing host cells under conditions for expression
31 of the gene.

32

1 The nucleic acid may be integrated into the genome
2 (e.g. chromosome) of the host cell. Integration may
3 be promoted by inclusion of sequences which promote
4 recombination with the genome in accordance with
5 standard techniques. The nucleic acid may be on an
6 extra-chromosomal vector within the cell, or
7 otherwise identifiably heterologous or foreign to
8 the cell.

9

10 **RNAi agents**

11

12 As described herein, c-FLIP inhibitors for use in
13 the invention may be RNAi agents.

14

15 RNA interference (RNAi) or posttranscriptional gene
16 silencing (PTGS) is a process whereby double-
17 stranded RNA induces potent and specific gene
18 silencing. RNAi is mediated by RNA-induced silencing
19 complex (RISC), a sequence-specific, multicomponent
20 nuclease that destroys messenger RNAs homologous to
21 the silencing trigger. RISC is known to contain
22 short RNAs (approximately 22 nucleotides) derived
23 from the double-stranded RNA trigger.

24

25 In one aspect, the invention provides methods of
26 employing an RNAi agent to modulate expression,
27 preferably reducing expression of a target gene, c-
28 FLIP, in a mammalian, preferably human host. By
29 reducing expression is meant that the level of
30 expression of a target gene or coding sequence is
31 reduced or inhibited by at least about 2-fold,
32 usually by at least about 5-fold, e.g., 10-fold, 15-

1 fold, 20-fold, 50-fold, 100-fold or more, as
2 compared to a control. In certain embodiments, the
3 expression of the target gene is reduced to such an
4 extent that expression of the c-FLIP gene /coding
5 sequence is effectively inhibited. By modulating
6 expression of a target gene is meant altering, e.g.,
7 reducing, translation of a coding sequence, e.g.,
8 genomic DNA, mRNA etc., into a polypeptide, e.g.,
9 protein, product.

10

11 The RNAi agents that may be employed in preferred
12 embodiments of the invention are small ribonucleic
13 acid molecules (also referred to herein as
14 interfering ribonucleic acids), that are present in
15 duplex structures, e.g., two distinct
16 oligoribonucleotides hybridized to each other or a
17 single ribooligonucleotide that assumes a small
18 hairpin formation to produce a duplex structure.
19 Preferred oligoribonucleotides are ribonucleic
20 acids of not greater than 100 nt in length,
21 typically not greater than 75 nt in length. Where
22 the RNA agent is an siRNA, the length of the duplex
23 structure typically ranges from about 15 to 30 bp,
24 usually from about 20 and 29 bps, most preferably 21
25 bp. Where the RNA agent is a duplex structure of a
26 single ribonucleic acid that is present in a hairpin
27 formation, i.e., a shRNA, the length of the
28 hybridized portion of the hairpin is typically the
29 same as that provided above for the siRNA type of
30 agent or longer by 4-8 nucleotides.

31

1 In certain embodiments, instead of the RNAi agent
2 being an interfering ribonucleic acid, e.g., an
3 siRNA or shRNA as described above, the RNAi agent
4 may encode an interfering ribonucleic acid. In these
5 embodiments, the RNAi agent is typically a DNA that
6 encodes the interfering ribonucleic acid. The DNA
7 may be present in a vector.

8
9 The RNAi agent can be administered to the host using
10 any suitable protocol known in the art. For example,
11 the nucleic acids may be introduced into tissues or
12 host cells by viral infection, microinjection,
13 fusion of vesicles, particle bombardment, or
14 hydrodynamic nucleic acid administration.

15
16 DNA directed RNA interference (ddRNAi) is an RNAi
17 technique which may be used in the methods of the
18 invention. ddRNAi is described in U.S. 6,573,099 and
19 GB 2353282. ddRNAi is a method to trigger RNAi
20 which involves the introduction of a DNA construct
21 into a cell to trigger the production of double
22 stranded (dsRNA), which is then cleaved into small
23 interfering RNA (siRNA) as part of the RNAi process.
24 ddRNAi expression vectors generally employ RNA
25 polymerase III promoters (e.g. U6 or H1) for the
26 expression of siRNA target sequences transfected in
27 mammalian cells. siRNA target sequences generated
28 from a ddRNAi expression cassette system can be
29 directly cloned into a vector that does not contain
30 a U6 promoter. Alternatively short single stranded
31 DNA oligos containing the hairpin siRNA target
32 sequence can be annealed and cloned into a vector

1 downstream of the pol III promoter. The primary
2 advantages of ddRNAi expression vectors is that they
3 allow for long term interference effects and
4 minimise the natural interferon response in cells..
5

6 **Antisense nucleic acids**
7

8 As described herein, c-FLIP inhibitors for use in
9 the invention may be anti-sense molecules or nucleic
10 acid constructs that express such anti-sense
11 molecules as RNA. The antisense molecules may be
12 natural or synthetic. Synthetic antisense molecules
13 may have chemical modifications from native nucleic
14 acids. The antisense sequence is complementary to
15 the mRNA of the targeted c-FLIP gene, and inhibits
16 expression of the targeted gene products. Antisense
17 molecules inhibit gene expression through various
18 mechanisms, e.g. by reducing the amount of mRNA
19 available for translation, through activation of
20 RNase H, or steric hindrance. One or a combination
21 of antisense molecules may be administered, where a
22 combination may comprise multiple different
23 sequences.
24

25 Antisense molecules may be produced by expression of
26 all or a part of the c-FLIP sequence in an
27 appropriate vector, where the transcriptional
28 initiation is oriented such that an antisense strand
29 is produced as an RNA molecule. Alternatively, the
30 antisense molecule may be a synthetic
31 oligonucleotide. Antisense oligonucleotides will
32 generally be at least about 7, usually at least

1 about 12, more usually at least about 16 nucleotides
2 in length, and usually not more than about 50,
3 preferably not more than about 35 nucleotides in
4 length.

5
6 A specific region or regions of the endogenous c-
7 FLIP sense strand mRNA sequence is chosen to be
8 complemented by the antisense sequence. Selection of
9 a specific sequence for the oligonucleotide may use
10 an empirical method, where several candidate
11 sequences are assayed for inhibition of expression
12 of the target gene in an in vitro or animal model. A
13 combination of sequences may also be used, where
14 several regions of the mRNA sequence are selected
15 for antisense complementation.

16
17 Antisense oligonucleotides may be chemically
18 synthesized by methods known in the art (see Wagner
19 et al. (1993), supra, and Milligan et al., supra.)
20 Preferred oligonucleotides are chemically modified
21 from the native phosphodiester structure, in order
22 to increase their intracellular stability and
23 binding affinity. A number of such modifications
24 have been described in the literature, which alter
25 the chemistry of the backbone, sugars or
26 heterocyclic bases. Among useful changes in the
27 backbone chemistry are phosphorodiamidate linkages,
28 methylphosphonates phosphorothioates;
29 phosphorodithioates, where both of the non-bridging
30 oxygens are substituted with sulfur;
31 phosphoroamidites; alkyl phosphotriesters and
32 boranophosphates. Achiral phosphate derivatives

1 include 3'-O-5'-S-phosphorothioate, 3'-S-5'-O-
2 phosphorothioate, 3'-CH₂-5'-O-phosphonate and 3'-NH-
3 5'-O-phosphoroamidate. Peptide nucleic acids may
4 replace the entire ribose phosphodiester backbone
5 with a peptide linkage. Sugar modifications may also
6 be used to enhance stability and affinity.

8 **Chemotherapeutic Agents**

9 Any suitable thymidylate synthase inhibitor,
10 platinum cytotoxic agent or topoisomerase inhibitor
11 may be used in the present invention. Examples of
12 thymidylate synthase inhibitors which may be used in
13 the methods of the invention include 5-FU, MTA and
14 TDX. In a preferred embodiment, the thymidylate
15 synthase inhibitor is 5-FU. Examples of platinum
16 cytotoxic agents which may be used include cisplatin
17 and oxaliplatin. In a particularly preferred
18 embodiment of the invention, the chemotherapeutic
19 agent is cisplatin. A topoisomerase inhibitor, which
20 may be used in the present invention is irenotecan
21 (CPT-11).

23 **Treatment**

24
25 "Treatment" includes any regime that can benefit a
26 human or non-human animal. The treatment may be in
27 respect of an existing condition or may be
28 prophylactic (preventative treatment). Treatment may
29 include curative, alleviation or prophylactic
30 effects.

31

1 "Treatment of cancer" includes treatment of
2 conditions caused by cancerous growth and includes
3 the treatment of neoplastic growths or tumours.
4 Examples of tumours that can be treated using the
5 invention are, for instance, sarcomas, including
6 osteogenic and soft tissue sarcomas, carcinomas,
7 e.g., breast-, lung-, bladder-, thyroid-, prostate-,
8 colon-, rectum-, pancreas-, stomach-, liver-,
9 uterine-, cervical and ovarian carcinoma, lymphomas,
10 including Hodgkin and non-Hodgkin lymphomas,
11 neuroblastoma, melanoma, myeloma, Wilms tumor, and
12 leukemias, including acute lymphoblastic leukaemia
13 and acute myeloblastic leukaemia, gliomas and
14 retinoblastomas.

15

16 In preferred embodiments of the invention, the
17 cancer is one or more of colorectal, breast ,
18 ovarian, cervical, gastric, lung, liver, skin and
19 myeloid (e.g. bone marrow) cancer.

20

21 **Administration**

22

23 As described above, c-FLIP inhibitors of and for use
24 in the present invention may be administered in any
25 suitable way. Moreover in any of the first to fifth
26 aspects of the invention, they may be used in
27 combination therapy with other treatments, for
28 example, other chemotherapeutic agents or binding
29 members. In such embodiments, the c-FLIP inhibitors
30 or compositions of the invention may be administered
31 simultaneously, separately or sequentially with
32 another chemotherapeutic agent.

1

2 Where administered separately or sequentially, they
3 may be administered within any suitable time period
4 e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of
5 each other. In preferred embodiments, they are
6 administered within 6, preferably within 2, more
7 preferably within 1, most preferably within 20
8 minutes of each other.

9

10 In a preferred embodiment, the c-FLIP inhibitors
11 and/or compositions of the invention are
12 administered as a pharmaceutical composition, which
13 will generally comprise a suitable pharmaceutical
14 excipient, diluent or carrier selected dependent on
15 the intended route of administration.

16

17 The c-FLIP inhibitors and/or compositions of the
18 invention may be administered to a patient in need
19 of treatment via any suitable route.

20

21 Some suitable routes of administration include (but
22 are not limited to) oral, rectal, nasal, topical
23 (including buccal and sublingual), vaginal or
24 parenteral (including subcutaneous, intramuscular,
25 intravenous, intradermal, intrathecal and epidural)
26 administration. Intravenous administration is
27 preferred.

28

29 The C-FLIP inhibitor, product or composition may be
30 administered in a localised manner to a tumour site
31 or other desired site or may be delivered in a
32 manner in which it targets tumour or other cells.

1 Targeting therapies may be used to deliver the
2 active agents more specifically to certain types of
3 cell, by the use of targeting systems such as
4 antibody or cell specific ligands. Targeting may be
5 desirable for a variety of reasons, for example if
6 the agent is unacceptably toxic, or if it would
7 otherwise require too high a dosage, or if it would
8 not otherwise be able to enter the target cells.

9
10 For intravenous, injection, or injection at the site
11 of affliction, the active ingredient will be in the
12 form of a parenterally acceptable aqueous solution
13 which is pyrogen-free and has suitable pH,
14 isotonicity and stability. Those of relevant skill
15 in the art are well able to prepare suitable
16 solutions using, for example, isotonic vehicles such
17 as Sodium Chloride Injection, Ringer's Injection,
18 Lactated Ringer's Injection. Preservatives,
19 stabilisers, buffers, antioxidants and/or other
20 additives may be included, as required.

21
22 Pharmaceutical compositions for oral administration
23 may be in tablet, capsule, powder or liquid form. A
24 tablet may comprise a solid carrier such as gelatin
25 or an adjuvant. Liquid pharmaceutical compositions
26 generally comprise a liquid carrier such as water,
27 petroleum, animal or vegetable oils, mineral oil or
28 synthetic oil. Physiological saline solution,
29 dextrose or other saccharide solution or glycols
30 such as ethylene glycol, propylene glycol or
31 polyethylene glycol may be included.

32

1 The c-FLIP inhibitors and/or compositions of the
2 invention may also be administered via microspheres,
3 liposomes, other microparticulate delivery systems
4 or sustained release formulations placed in certain
5 tissues including blood. Suitable examples of
6 sustained release carriers include semipermeable
7 polymer matrices in the form of shared articles,
8 e.g. suppositories or microcapsules. Implantable or
9 microcapsular sustained release matrices include
10 polylactides (US Patent No. 3, 773, 919; EP-A-
11 0058481) copolymers of L-glutamic acid and gamma
12 ethyl-L-glutamate (Sidman et al, Biopolymers 22(1):
13 547-556, 1985), poly (2-hydroxyethyl-methacrylate)
14 or ethylene vinyl acetate (Langer et al, J. Biomed.
15 Mater. Res. 15: 167-277, 1981, and Langer, Chem.
16 Tech. 12:98-105, 1982). Liposomes containing the
17 polypeptides are prepared by well-known methods: DE
18 3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692,
19 1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980;
20 EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-
21 0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos
22 4,485,045 and 4,544,545. Ordinarily, the liposomes
23 are of the small (about 200-800 Angstroms)
24 unilamellar type in which the lipid content is
25 greater than about 30 mol. % cholesterol, the
26 selected proportion being adjusted for the optimal
27 rate of the polypeptide leakage.

28

29 Examples of the techniques and protocols mentioned
30 above and other techniques and protocols which may
31 be used in accordance with the invention can be

1 found in Remington's Pharmaceutical Sciences, 16th
2 edition, Oslo, A. (ed), 1980.

3

4

5 **Pharmaceutical Compositions**

6

7 Pharmaceutical compositions according to the present
8 invention, and for use in accordance with the
9 present invention may comprise, in addition to
10 active ingredients, a pharmaceutically acceptable
11 excipient, carrier, buffer stabiliser or other
12 materials well known to those skilled in the art.
13 Such materials should be non-toxic and should not
14 interfere with the efficacy of the active
15 ingredient. The precise nature of the carrier or
16 other material will depend on the route of
17 administration, which may be oral, or by injection,
18 e.g. intravenous.

19

20 The formulation may be a liquid, for example, a
21 physiologic salt solution containing non-phosphate
22 buffer at pH 6.8-7.6, or a lyophilised powder.

23

24 **Dose**

25

26 The c-FLIP inhibitors or compositions of the
27 invention are preferably administered to an
28 individual in a "therapeutically effective amount",
29 this being sufficient to show benefit to the
30 individual. The actual amount administered, and
31 rate and time-course of administration, will depend
32 on the nature and severity of what is being treated.

1 Prescription of treatment, e.g. decisions on dosage
2 etc, is ultimately within the responsibility and at
3 the discretion of general practitioners and other
4 medical doctors, and typically takes account of the
5 disorder to be treated, the condition of the
6 individual patient, the site of delivery, the method
7 of administration and other factors known to
8 practitioners.

9

10

11 **Brief Description of the Figures**

12

13 The invention will now be described further in the
14 following non-limiting examples. Reference is made
15 to the accompanying drawings in which:

16

17 Figure 1A illustrates Western blot analysis of Fas,
18 FasL, procaspase 8, FADD, BID, Bcl-2, c-FLIP_L, c-
19 FLIP_S, DcR3 and β -tubulin in MCF-7 cells 72 hours
20 after treatment with 5 μ M 5-FU and 50nM TDX.

21

22 Figure 1B illustrates analysis of the interaction
23 between Fas and FasL following treatment with 5 μ M 5-
24 FU and 50nM TDX for 48 hours. Lysates were
25 immunoprecipitated using a FasL polyclonal antibody
26 and analysed by Western blot using a Fas monoclonal
27 antibody.

28

29 Figure 1C illustrates analysis of the interaction
30 between Fas and p43- c-FLIP_L following treatment
31 with 5 μ M 5-FU and 50nM TDX for 48 hours. Lysates
32 were immunoprecipitated using the anti-Fas CH-11

1 monoclonal antibody and analysed by Western blot
2 using a c-FLIP monoclonal antibody.

3
4 Figure 2A illustrates flow cytometry of MCF-7 cells
5 treated with no drug (control), CH-11 alone
6 (250ng/ml), 5-FU alone (5 μ M) for 96 hours, or co-
7 treated with 5-FU for 72 hours followed by CH-11 for
8 a further 24 hours.

9
10 Figure 2B illustrates flow cytometry of MCF-7 cells
11 treated with no drug (control), CH-11 alone
12 (250ng/ml), TDX alone (50nM) for 96 hours, or co-
13 treated with TDX for 72 hours followed by CH-11 for
14 a further 24 hours.

15
16 Figure 2C illustrates Western blot analysis of Fas
17 expression in MCF-7 cells treated with 5 μ M 5-FU for
18 48 hours. β -tubulin was assessed as a loading
19 control.

20
21 Figure 2D illustrates flow cytometry of MCF-7 cells
22 treated with no drug (control), CH-11 alone
23 (250ng/ml), OXA alone (5 μ M) for 96 hours, or co-
24 treated with OXA for 72 hours followed by CH-11 for
25 a further 24 hours.

26
27 Figure 2E illustrates Western blot analysis of Fas,
28 procaspase 8 and PARP expression in MCF-7 cells
29 treated with 5 μ M 5-FU alone for 96 hours, or co-
30 treated with 5-FU for 72 hours followed by CH-11 for
31 a further 24 hours.

32

1 Figure 2F illustrates Western blot analysis
2 examining the kinetics of caspase 8 activation and
3 c-FLIP_L processing in MCF-7 cells treated for 72
4 hours with 5 μ M 5-FU followed by 250ng/ml CH-11 for
5 the indicated times.

6
7 Figure 3A illustrates Western blot analysis of Fas
8 expression in HCT116 cells treated with 5-FU, TDX or
9 OXA for 48 hours. Equal loading was assessed using a
10 β -tubulin antibody.

11
12 Figure 3B illustrates Western blot analysis of
13 procaspase 8 and PARP expression in HCT116 cells
14 treated no drug (Con), 5 μ M 5-FU, 100nM TDX or 2 μ M
15 OXA in the presence or absence of co-treatment with
16 200ng/ml CH-11. For each combined treatment the
17 cells were pre-treated with chemotherapeutic drug
18 for 24 hours followed by CH-11 for a further 24
19 hours.

20
21 Figure 4A illustrates Western blot of c-FLIP_L
22 expression in MCF-7 cells stably transfected with a
23 FLIPL (FL) construct or empty vector (EV).

24
25 Figure 4B illustrates MTT cell viability assays in
26 EV68, FL44 and FL64 cells treated with 5 μ M 5-FU in
27 combination with 250ng/ml CH-11. The combined
28 treatment resulted in a synergistic decrease in cell
29 viability in EV68 cells (RI=2.06), but not FL44
30 (RI=1.14) or FL64 (1.01) cells.

31

1 Figure 4C illustrates Western blot analysis of c-
2 FLIP_L, procaspase 8 and PARP expression in EV68 and
3 FL64 cells treated with no drug (Con) or 5 μ M 5-FU in
4 the presence (+) or absence (-) of co-treatment with
5 250ng/ml CH-11. For each combined treatment, the
6 cells were pre-treated with 5-FU for 72 hours
7 followed by CH-11 for a further 24 hours.

8
9 Figure 5A illustrates MTT cell viability assays in
10 EV68, FL44 and FL64 cells treated with 50nM TDX or
11 500nM MTA in the presence and absence of 250ng/ml
12 CH-11. Combined TDX/CH-11 treatment resulted in a
13 synergistic decrease in cell viability in EV68 cells
14 (RI=1.75), that was significantly reduced in FL44
15 (RI=1.22) or FL64 (RI=1.19) cells. Combined MTA/CH-
16 11 treatment resulted in a synergistic decrease in
17 cell viability in EV68 cells (RI=1.86), that was
18 significantly reduced in FL44 (RI=1.29) and FL64
19 (RI=1.06) cells.

20
21 Figure 5B illustrates MTT cell viability assays in
22 EV68, FL44 and FL64 cells treated with 2.5 μ M OXA in
23 the presence and absence of 250ng/ml CH-11. Combined
24 OXA/CH-11 treatment resulted in a synergistic
25 decrease in cell viability in EV68 cells (RI=2.13),
26 that was significantly reduced in FL64 (RI=1.22) or
27 FL44 (1.19) cells.

28
29 Figure 5C Western blot analysis of procaspase 8 and
30 PARP expression in EV68 and FL64 cells treated with
31 50nM TDX or 500nM MTA in the presence (+) or absence
32 (-) of co-treatment with 250ng/ml CH-11.

1

2 Figure 5D illustrates Western blot analysis of
3 procaspase 8 and PARP expression in EV68 and FL64
4 cells treated with 2.5 μ M OXA in the presence (+) or
5 absence (-) of co-treatment with 250ng/ml CH-11. For
6 each combined treatment, the cells were pre-treated
7 with 5-FU for 72 hours followed by CH-11 for a
8 further 24 hours.

9

10 Figure 6A illustrates c-FLIP_L and c-FLIP_S expression
11 in HCT116 cells transfected with 0, 1 and 10nM FLIP-
12 targeted siRNA for 48 hours. Equal loading was
13 assessed using a β -tubulin antibody.

14

15 Figure 6B illustrates MTT cell viability assays of
16 HCT116 cells transfected with 5nM FLIP-targeted (FT)
17 or scrambled control (SC) siRNA in the presence and
18 absence of co-treatment with 5 μ M 5-FU. Combined
19 treatment with 5-FU and FT siRNA resulted in a
20 synergistic decrease in cell viability (RI=1.92,
21 $p<0.0005$). No synergistic decrease in viability was
22 observed in cells co-treated with 5-FU and SC siRNA
23 (RI=0.98).

24

25 Figure 6C illustrates Western blot analysis of
26 caspase 8 activation and PARP cleavage in HCT116
27 cells 48 hours after treatment with no drug, 5 μ M 5-
28 FU or 100nM TDX in mock transfected cells (M), cells
29 transfected with 1nM scrambled control (SC) and
30 cells transfected with 1nM FLIP-targeted (FT) siRNA.

31

1 Figure 7A illustrates c-FLIP_L and c-FLIP_S expression
2 in MCF-7 cells transfected with 10nM FLIP-targeted
3 (FT) or scrambled control (SC) siRNA for 48 hours.
4 Equal loading was assessed using a β -tubulin
5 antibody.

6
7 Figure 7B illustrates MTT cell viability assays of
8 MCF-7 cells transfected with 2.5nM FT siRNA in the
9 presence and absence of co-treatment with 5 μ M 5-FU.
10 The combined treatment resulted in a synergistic
11 decrease in cell viability (RI=1.56, p<0.005).
12 Figure 7C Western blot analysis of PARP cleavage in
13 MCF-7 cells 96 hours after treatment with 5-FU in
14 the presence (+) and absence (-) of 10nM FLIP-
15 targeted siRNA.

16
17 Figure 8 illustrates MTT cell viability assays of
18 HCT116 cells transfected with 0.5nM FT or SC siRNA
19 in the presence and absence of co-treatment with:
20 Fig 8A 5 μ M 5-FU; Fig 8B 100nM TDX and Fig 8C 1 μ M
21 OXA. Cells were assayed after 72 hours. Combined
22 treatment with FT siRNA (but not SC siRNA) and each
23 cytotoxic drug resulted in synergistic decreases in
24 cell viability as indicated by the RI values
25 (p<0.0005 for each combination).

26
27 Figure 9 illustrates: A Western blot analysis of Fas
28 expression in p53 wild type HCT116 cells treated
29 with 5-FU or oxaliplatin (OXA) for 48 hours. B
30 Western blot analysis of caspase 8 activation, PARP
31 cleavage and c-FLIP expression in p53 wild type
32 HCT116 cells treated with no drug (Con), 5 μ M 5-FU,

1 or 1 μ M OXA in the presence or absence of co-
2 treatment with 200ng/mL CH-11. For each combined
3 treatment the cells were pre-treated with
4 chemotherapeutic drug for 24 hours followed by CH-11
5 for a further 24 hours.

6
7 Figure 10 illustrates: A c-FLIP_L and c-FLIP_S
8 expression in HLacZ, HFL17, HFL24, HFS19 and HFS44
9 cell lines. B Flow cytometric analysis of cell cycle
10 arrest and apoptosis in HLacZ, HFL17, HFL24, HFS19
11 and HFS44 cell lines 72 hours after treatment with
12 5 μ M 5-FU, 1 μ M oxaliplatin (OXA) and 5 μ M CPT-11. C
13 Flow cytometric analysis of HLacZ, HFL17, HFL24,
14 HFS19 and HFS44 cells after co-treatment with
15 50ng/mL CH-11 and 2.5 μ M 5-FU, 0.5 μ M oxaliplatin
16 (OXA) and 1 μ M CPT-11. For each combined treatment
17 the cells were pre-treated with chemotherapeutic
18 drug for 24 hours followed by CH-11 for a further 24
19 hours.

20
21 Figure 11 illustrates: A c-FLIP_L and c-FLIP_S
22 expression in p53 wild type HCT116 cells transfected
23 with 1nM control siRNA (SC) and 1nM FLIP-targeted
24 (FT) siRNA for 24 hours. B Flow cytometric analysis
25 of apoptosis in HCT116 cells transfected with 0.5nM
26 FT or 0.5nM SC siRNA. Transfected cells were co-
27 treated with no drug, 5 μ M 5-FU, or 1 μ M oxaliplatin
28 (OXA) for 48 hours. C (Panel 1) Western blot
29 analysis of caspase 8 activation and PARP cleavage
30 in HCT116 cells 48 hours after treatment of mock
31 transfected cells (M), cells transfected with 0.5nM
32 SC and cells transfected with 0.5nM FT siRNA with no

1 drug, 5 μ M 5-FU or 100nM TDX. (Panel 2) Caspase 8
2 activation and PARP cleavage in HCT116 cells
3 transfected with 0.5nM SC or 0.5nM FT siRNA and
4 treated with no drug, or 1 μ M oxaliplatin (OXA) for
5 24 hours. (Panel 3) Caspase 8 activation and PARP
6 cleavage in HCT116 cells after transfection with
7 0.5nM SC or 0.5nM FT siRNA and treatment with no
8 drug, 2.5 μ M or 5 μ M CPT-11 for 24 hours. **D** MTT cell
9 viability assays in HCT116p53^{+/+} cells transfected
10 with FT siRNA and co-treated with 5-FU, oxaliplatin
11 (OXA) and CPT-11. Cell viability was assayed after
12 72 hours. The nature of the interaction between the
13 chemotherapeutic drugs and FT siRNA was determined
14 by calculating the combination index (CI) according
15 to the method of Chou and Talalay. CI values of 1,
16 <1, and >1 indicate additive, synergistic and
17 antagonistic effects respectively. Results are
18 representative of at least 3 separate experiments.

19
20 Figure 12 illustrates: **A** Western blot analysis of c-
21 FLIP_L and c-FLIP_S expression in p53 wild type (wt)
22 and null HCT116 cells. **B** Western blot analysis of c-
23 FLIP_L and c-FLIP_S expression in HCT116p53^{-/-} cells
24 transfected with 1nM control siRNA (SC) and 1nM
25 FLIP-targeted (FT) siRNA for 24 hours. **C** Flow
26 cytometric ananlysis of apoptosis in HCT116p53^{-/-}
27 cells transfected with 1nM FT or 1nM SC siRNA.
28 Transfected cells were co-treated with no drug, 5 μ M
29 5-FU, 5 μ M oxaliplatin (OXA) or 1 μ M CPT-11 for 72
30 hours. **D** MTT cell viability assays in HCT116p53^{-/-}
31 cells transfected with FT siRNA and co-treated with
32 5-FU, oxaliplatin (OXA), and CPT-11. Cell viability

1 was assayed after 72 hours. The nature of the
2 interaction between the chemotherapeutic drugs and
3 FLIP-targeted siRNAs was determined by calculating
4 the combination index (CI) according to the method
5 of Chou and Talalay. Results are representative of
6 at least 3 separate experiments.

7
8 Figure 13 illustrates: **A** c-FLIP_L and c-FLIP_S
9 expression in RKO and H630 cells transfected with
10 1nM control siRNA (SC) and 1nM FLIP-targeted (FT)
11 siRNA for 24 hours. **B** Flow cytometric analysis of
12 apoptosis in RKO cells transfected with 2.5nM FT or
13 2.5nM SC siRNA and H630 cells transfected with 1nM
14 FT or 1nM SC siRNA. SiRNA-transfected RKO cells were
15 co-treated with no drug, 5µM 5-FU, 1µM oxaliplatin
16 (OXA) or 2.5µM CPT-11 for 72 hours. SiRNA-
17 transfected H630 cells were co-treated with no drug,
18 5µM 5-FU, 2.5µM oxaliplatin (OXA) or 1µM CPT-11 for
19 72 hours. **C** MTT cell viability assays in RKO and
20 H630 cells transfected with FT siRNA and co-treated
21 with 5-FU, oxaliplatin (OXA), and CPT-11. Cell
22 viability was assayed after 72 hours. The nature of
23 the interaction between the chemotherapeutic drugs
24 and FLIP-targeted siRNAs was determined by
25 calculating the combination index (CI) according to
26 the method of Chou and Talalay. Results are
27 representative of at least 3 separate experiments.

28
29 Figure 14 illustrates: **A** MTT cell viability assays
30 in HCT116p53^{+/+} cells transfected with FT or SC siRNA
31 for 72 hours. **B** Western blot analysis of c-FLIP
32 expression and PARP cleavage in p53 wild type

1 (p53^{+/+}) and p53 null (p53^{-/-}) HCT116 cells 24 hours
2 after transfection with 0, 1 and 10nM FT siRNA. **C**
3 Flow cytometric analysis of apoptosis in p53 wild
4 type (p53^{+/+}) and p53 null (p53^{-/-}) HCT116 cells
5 transfected with FT or SC siRNA for 48 hours. **D**
6 Apoptosis in HCT116p53^{-/-} cells transfected with FT
7 siRNA for 48 and 72 hours. **E** Apoptosis in RKO cells
8 transfected with FT or SC siRNA for 72 hours. **F**
9 Apoptosis in H630 cells transfected with FT or SC
10 siRNA for 72 hours.

11

12 Figure 15 illustrates: **A** Kinetics of c-FLIP down-
13 regulation, caspase 8 activation and PARP cleavage
14 in HCT116p53^{+/+} cells transfected with 0, 1 and 10nM
15 FT siRNA. **B** Flow cytometric analysis of the kinetics
16 of apoptosis induction in HCT116p53^{+/+} cells
17 transfected with 10nM FT or 10nM SC siRNA.

18

19 Figure 16 illustrates: **A** c-FLIP_L and c-FLIP_S
20 expression and PARP cleavage in p53 wild type HCT116
21 cells transfected with 10nM control siRNA (SC) and
22 10nM FLIP_L-specific (FL) siRNA for 24 hours. **B**
23 Western blot analysis of PARP cleavage in HCT116
24 cells transfected with 0.5nM SC or 0.5nM FL siRNA
25 and treated with no drug, 1μM oxaliplatin (OXA) or
26 2.5μM for 24 hours, or 5μM 5-FU for 48 hours. **C** MTT
27 cell viability assays in HCT116p53^{+/+} cells
28 transfected with FL siRNA and co-treated with 5-FU
29 oxaliplatin (OXA), and CPT-11. Cell viability was
30 assayed after 72 hours. The nature of the
31 interaction between the chemotherapeutic drugs and
32 FLIP-targeted siRNAs was determined by calculating

1 the combination index (CI) according to the method
2 of Chou and Talalay. Results are representative of
3 at least 3 separate experiments.

4
5 Figure 17 illustrates illustrates graphs of RI
6 values calculated from MTT cell viability assays of
7 the chemotherapeutic agents 5-FU, Tomudex (TDX),
8 CPT-11 and Oxaliplatin used in combination with the
9 agonistic anti-Fas antibody CH-11 (200ng/ml). The RI
10 is calculated as ratio of the expected cell survival
11 (S_{exp} , defined as the product of the survival
12 observed with drug A alone and the survival observed
13 with drug B alone) to the observed cell survival
14 (S_{obs}) for the combination of A and B
15 ($RI = S_{exp}/S_{obs}$). Synergism is defined as an RI
16 greater than 1.

17
18 Figure 18 illustrates A, Flow cytometry analysis of
19 cells stained with propidium iodide stained HCT116
20 p53 wild-type and null cells treated with 5-FU
21 (5 μ M), TDX (50nM), CPT-11 (5 μ M) and Oxaliplatin (1 μ M)
22 for 24 hours and then with CH-11 (50ng/ml) for an
23 additional 24 hours. B, Sub G0/G1 populations for
24 the HCT116p53 wild-type and null cell lines treated
25 with chemotherapy drugs with and without CH-11 50
26 ng/ml.

27
28 Figure 19 illustrates the effect of adding CH-11
29 200ng/ml for 24 hours to HCT116 p53 wild-type and
30 null cells already treated for 24 hours with 5-FU
31 (5 μ M), CPT-11 (5 μ M) and Oxaliplatin (1 μ M) on PARP

1 cleavage and activation of procaspase 8 by Western
2 blot analysis.

3

4

5 **Examples**

6

7 **MATERIALS AND METHODS**

8 **Cell Culture.** All cells were maintained in 5% CO₂ at
9 37°C. MCF-7 cells were maintained in DMEM with 10%
10 dialyzed bovine calf serum supplemented with 1mM
11 sodium pyruvate, 2mM L-glutamine and 50µg/ml
12 penicillin/streptomycin (from Life Technologies
13 Inc., Paisley, Scotland). HCT116p53^{+/+} and HCT116p53^{-/-}
14 isogenic human colorectal cancer cells were kindly
15 provided by Professor Bert Vogelstein (John Hopkins
16 University, Baltimore, MD). HCT116 cells were grown
17 in McCoy's 5A medium (GIBCO) supplemented with 10%
18 dialysed foetal calf serum, 50mg/ml penicillin-
19 streptomycin, 2mM L-glutamine and 1mM sodium
20 pyruvate. Stably transfected MCF-7 and HCT116 cell
21 lines and 'mixed populations' of transfected cells
22 were maintained in medium supplemented with 100µg/ml
23 (MCF-7) or 1.5mg/ml (HCT116) G418 (from Life
24 Technologies Inc).

25

26 **Generation of c-FLIP overexpressing cell lines.** c-
27 FLIP_L and c-FLIP_S coding regions were PCR amplified
28 and ligated into the pcDNA/V5-His TOPO vector
29 according to the manufacturer's instructions (Life
30 Technologies Inc.). HCT116p53^{+/+} cells were co-
31 transfected with 10µg of each c-FLIP expression
32 construct and 1µg of a construct expressing a

1 puromycin resistance gene (pIRESpuro3, Clontech)
2 using GeneJuice. Stably transfected HCT116 cells
3 were selected and maintained in medium supplemented
4 with 1µg/ml puromycin (Life Technologies Inc.).
5 Stable overexpression of c-FLIP was assessed by
6 Western blot analysis.

7
8 **Western Blotting.** Western blots were performed as
9 previously described (Longley et al., 2002). The
10 Fas/CD95, Bcl-2 and BID (Santa Cruz Biotechnology,
11 Santa Cruz, CA), caspase 8 (Oncogene Research
12 Products, Darmstadt, Germany), PARP (Pharmingen, BD
13 Biosciences, Oxford, England), c-FLIP (NF-6, Alexis,
14 Bingham UK) DcR3 (Imgenex, San Diego, CA) mouse
15 monoclonal antibodies were used in conjunction with
16 a horseradish peroxidase (HRP)-conjugated sheep
17 anti-mouse secondary antibody (Amersham, Little
18 Chalfont, Buckinghamshire, England). FasL rabbit
19 polyclonal antibody (Santa Cruz Biotechnology) was
20 used in conjunction with an HRP-conjugated donkey
21 anti-rabbit secondary antibody (Amersham). Equal
22 loading was assessed using a β -tubulin mouse
23 monoclonal primary antibody (Sigma).

24
25 **Co-immunoprecipitation reactions.** 250µl of Protein A
26 (IgG) or Protein L (IgM) Sepharose beads (Sigma) and
27 1µg of the appropriate antibody were mixed at 4°C
28 for 1 hour. Antibody-associated beads were washed
29 three times with ELB buffer (250mM NaCl, 0.1%
30 IPEGAL, 5mM EDTA, 0.5mM DTT, 50mM HEPES). Protein
31 lysate (200-400µg) was then added, and the mixture
32 rotated at 4°C for 1 hour. The beads were then

1 washed in ELB buffer five times and resuspended in
2 100µl of Western sample buffer (250mM TRIS pH 6.8,
3 4% SDS, 2% glycerol, 0.02% bromophenol blue)
4 containing 10% β-mercaptoethanol. The samples were
5 then heated at 95°C for 5 minutes and centrifuged
6 (5mins/4,000rpm/4°C). The supernatant was collected
7 and analysed by Western blotting.

8
9 **Cell Viability Assays.** Cell viability was assessed
10 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-
11 diphenyltetrazolium bromide, Sigma) assay (Mosmann,
12 1983). To investigate drug-induced Fas-mediated
13 apoptosis, cells were seeded at 2,000-5,000 cells
14 per well on 96-well plates. After 24 hours, the
15 cells were treated with a range of concentrations of
16 5-FU, TDX, MTA or OXA for 24-72 hours followed by
17 the agonistic Fas monoclonal antibody, CH-11 (MBL,
18 Watertown, MA) for a further 24-48 hours. To assess
19 chemotherapy/siRNA interactions, 20,000-50,000 cells
20 were seeded per well on 24-well plates. Twenty-four
21 hours later, the cells were transfected with FLIP-
22 targeted (FT) or scrambled siRNA (SC). Four hours
23 after transfection, the cells were treated with a
24 range of concentrations of each drug for a further
25 72-96 hours. MTT (0.5mg/ml) was added to each well
26 and the cells were incubated at 37°C for a further 2
27 hours. The culture medium was removed and formazan
28 crystals reabsorbed in 200µl (96-well) or 1ml (24-
29 well) DMSO. Cell viability was determined by reading
30 the absorbance of each well at 570nm using a
31 microplate reader (Molecular Devices, Wokingham,
32 England).

1
2 **Flow Cytometric Analysis.** Cells were seeded at 1×10^5
3 per well of a 6-well tissue culture plate. After 24
4 hours, 5-FU, TDX or OXA was added to the medium and
5 the cells cultured for a further 72 hours, after
6 which time 250ng/ml CH-11 was added for 24 hours.
7 DNA content of harvested cells was evaluated after
8 propidium iodide staining of cells using the EPICS
9 XL Flow Cytometer (Coulter, Miami, Fl).

10
11 **siRNA transfections.** FLIP-targeted siRNA was
12 designed using the Ambion siRNA target finder and
13 design tool
14 (www.ambion.com/techlib/misc/siRNA_finder.html) to
15 inhibit both splice variants of c-FLIP. Both c-FLIP-
16 targeted (FT) and scrambled control (SC) siRNA were
17 obtained from Xeragon (Germantown, MD). The FT siRNA
18 sequence used was: AAG CAG TCT GTT CAA GGA GCA. The
19 FL siRNA sequence used was: AAG GAA CAG CTT GGC GCT
20 CAA. The control non-silencing siRNA sequence (SC)
21 used was: AAT TCT CCG AAC GTG TCA CGT. siRNA
22 transfections were performed on sub-confluent cells
23 incubated in Optimem medium using the oligofectamine
24 reagent (both from Life Technologies Inc) according
25 to the manufacturer's instructions.

26
27 **Statistical Analyses.** The nature of the interaction
28 between the chemotherapeutic drugs and FLIP-targeted
29 siRNAs was determined by calculating the combination
30 index (CI) according to the method of Chou and
31 Talalay (14). CI values were calculated from
32 isobolograms generated using the CalcuSyn software

1 programme (Microsoft Windows). According to the
2 definitions of Chou and Talalay, a CI value of 0.85-
3 0.9 is slightly synergistic, 0.7-0.85 is moderately
4 synergistic, 0.3-0.7 is synergistic and 0.1-0.3 is
5 strongly synergistic. An unpaired two-tailed t test
6 was used to determine the significance of changes in
7 levels of apoptosis between different treatment
8 groups.

9

10 RESULTS

11

12 **Example 1. c-FLIP_L is up-regulated, processed and**
13 **bound to Fas in response to 5-FU and TDX.**

14

15 Analysis of Fas expression in MCF-7 cells revealed
16 that it was up-regulated by ~12-fold 72 hours after
17 treatment with an IC60 dose 5-FU and was also highly
18 up-regulated (by ~7-fold) in response to treatment
19 with an IC60 dose (25nM) of TDX (Fig. 1A). FasL
20 expression was unaffected by each drug treatment,
21 but appeared to be highly expressed in these cells.
22 Expression of FADD was also unaffected by drug
23 treatment. Somewhat surprisingly, neither caspase 8,
24 nor its substrate BID were activated in 5-FU- or
25 TDX-treated cells as indicated by a lack of down-
26 regulation of the levels of procaspase 8 or full-
27 length BID (Fig. 1A). Bcl-2 was highly down-
28 regulated in response to each agent. Interestingly,
29 c-FLIP_L but not c-FLIP_S was up-regulated by drug
30 treatment. Furthermore, c-FLIP_L was processed to its
31 p43-form indicative of its recruitment and
32 processing at the DISC (Fig. 1A). Expression of the

1 Fas decoy receptor DcR3 was unaltered by drug
2 treatment in these cells.

3
4 To further investigate the apparent inhibition of
5 caspase 8 activation in 5-FU- and TDX-treated cells,
6 we analysed the interaction between Fas and FasL
7 following drug treatment. Co-immunoprecipitation
8 reactions demonstrated that there was increased Fas-
9 FasL binding following drug treatment (Fig. 1B),
10 suggesting that the inhibition of caspase 8
11 activation was occurring downstream of receptor
12 ligation. In support of this, we found that drug
13 treatment increased the interaction between Fas and
14 p43- c-FLIP_L (Fig. 1C). These results suggested the
15 involvement of c-FLIP_L in inhibiting drug-induced
16 activation of Fas-mediated apoptosis in MCF-7 cells.

17
18 **Example 2 Activation of drug-induced apoptosis by**
19 **the Fas-targeted antibody CH-11 coincides with**
20 **processing of c-FLIP_L.** Expression of FasL by
21 activated T cells and NK cells induces apoptosis of
22 Fas expressing target cells in vivo. To mimic the
23 effects of these immune effector cells in vitro, the
24 agonistic Fas monoclonal antibody CH-11 was used.
25 Cells were treated with either 5-FU or TDX for 72
26 hours followed by 250ng/ml CH-11 treatment for 24
27 hours. We found that CH-11 alone had little effect
28 on apoptosis (Figs. 2A and B). Treatment with 5-FU
29 alone for 96 hours resulted in a modest ~2-fold
30 induction of apoptosis in response to 5µM 5-FU (Fig.
31 2A). However, addition of CH-11 to 5-FU-treated
32 cells resulted in a dramatic increase in apoptosis,

1 with a ~55% of cells in the sub-G1/G0 apoptotic
2 phase following co-treatment with 5 μ M 5-FU and CH-
3 11. Similarly, the combination of TDX with CH-11
4 resulted in dramatic activation of apoptosis, with
5 ~60% of cells in the sub-G1/G0 apoptotic phase
6 following combined treatment with 25nM TDX and CH-11
7 (Fig. 2B). We also examined the effect of CH-11 on
8 apoptosis induced by the DNA-damaging agent OXA,
9 which also potently induces Fas expression in MCF-7
10 cells (Fig. 2C). Similar to its effect on 5-FU and
11 TDX-treated cells, CH-11 induced apoptosis of OXA-
12 treated cells, with ~50% of cells in the sub-G1/G0
13 apoptotic phase (Fig. 2D).

14
15 We subsequently analysed activation of the Fas
16 pathway in MCF-7 cells following co-treatment with
17 5-FU and CH-11. As already noted, treatment with 5-
18 FU alone resulted in dramatic up-regulation of Fas,
19 but had no effect on caspase 8 activation (Fig. 2E).
20 However, co-treatment of MCF-7 cells with 5-FU and
21 CH-11 resulted in a dramatic activation of caspase 8
22 as indicated by complete loss of procaspase 8 (Fig.
23 2E). Furthermore, cleavage of PARP (poly(ADP) ribose
24 polymerase), a hallmark of apoptosis, was only
25 observed in MCF-7 cells co-treated with 5-FU and CH-
26 11 (Fig. 2E). We next analysed the kinetics of
27 caspase 8 activation in 5-FU and CH-11 co-treated
28 cells. Caspase 8 was potently activated 12 hours
29 after addition of CH-11 to 5-FU pre-treated cells
30 (Fig. 2F). Importantly, this coincided with complete
31 processing of c-FLIP_L to its p43-form (Fig. 2F). By
32 24 hours after the addition of CH-11, neither

1 procaspase 8 nor c-FLIP_L (both its full-length and
2 truncated forms) was detected.

3
4 Similarly, treatment of HCT116p53^{+/+} cells with
5 IC₅₀(72h) doses of 5-FU (5μM) or oxaliplatin (1μM) for
6 48 hours resulted in potent up-regulation of Fas
7 expression (Fig. 8A), but only modest activation of
8 caspase 8 and no PARP cleavage (Fig. 8B). However,
9 co-treatment with each drug and CH-11 resulted in
10 potent activation of caspase 8 and PARP cleavage
11 (Fig. 8B). Activation of caspase 8 correlated with
12 the complete processing of c-FLIP_L to p43-FLIP_L in
13 drug and CH-11 co-treated cells (Fig. 8B).
14 Furthermore, addition of CH-11 to 5-FU- and
15 oxaliplatin-treated HCT116p53^{+/+} cells resulted in
16 ~4- and ~8-fold up-regulation of c-FLIP_S
17 respectively (Fig. 8B). These results suggested the
18 involvement of c-FLIP in regulating Fas-mediated
19 apoptosis in HCT116p53^{+/+} cells following
20 chemotherapy.

21
22 We also examined the ability of CH-11 to activate
23 apoptosis in the HCT116 colon cancer cell line. Fas
24 was potently up-regulated in HCT116 cells 48 hours
25 after treatment with 5-FU, TDX and OXA (Fig. 3A).
26 Treatment with each drug alone or CH-11 alone for 48
27 hours failed to significantly activate caspase 8 or
28 induce PARP cleavage (Fig. 3B). However, treatment
29 with each drug for 24 hours followed by CH-11 for a
30 further 24 hours resulted in activation of caspase 8
31 and PARP cleavage. Importantly, activation of
32 caspase 8 correlated with processing of c-FLIP_L in

1 drug and CH-11 co-treated cells (Fig. 3B).

2

3 To further test the hypothesis that the
4 intracellular signal to commit to death receptor-
5 mediated apoptosis in HCT116p53^{+/+} cells following
6 drug treatment was regulated by c-FLIP, the
7 inventors generated HCT116p53^{+/+} cell lines that
8 overexpressed c-FLIP_L or c-FLIP_S. The HFL17 and HFL24
9 cell lines both overexpressed c-FLIP_L by ~6-fold
10 compared to cells transfected with a LacZ-expressing
11 construct (HLacZ), while the HFS19 and HFS44 cell
12 lines overexpressed c-FLIP_S by ~5- and ~10-fold
13 respectively compared to the control cell line (Fig.
14 9A). Growth inhibition studies (MTT assays) were
15 carried out to determine the IC_{50(72h)} dose for each
16 chemotherapy in each cell line. It was found that
17 overexpressing c-FLIP_S had no significant effect on
18 the IC_{50(72h)} dose of any of the drugs, while c-FLIP_L
19 overexpression caused a moderate 1.7-2.0-fold
20 increase in the IC_{50(72h)} dose of oxaliplatin, but had
21 no effect on the IC_{50(72h)} doses of the other drugs
22 (Table 1).

23

24 Flow cytometry revealed that c-FLIP_L overexpression
25 did not affect cell cycle arrest in response to
26 chemotherapy, but had a marked effect on
27 chemotherapy-induced apoptosis (Fig. 9B). For
28 example, treatment with 5μM 5-FU for 72 hours
29 resulted in cell cycle arrest at the G1/S phase
30 boundary in each cell line, however the levels of
31 apoptosis in the two c-FLIP_L-overexpressing lines
32 was significantly reduced compared to the control

1 cell line, with ~15% of HFL17 cells and ~17% of
2 HFL24 cells in the sub-G₁/G₀ apoptotic fraction
3 compared to ~41% in the HLacZ cell line ($p < 0.0001$,
4 Fig. 9B). In contrast, the levels of apoptosis
5 induced by 5-FU in the two c-FLIP_S-overexpressing
6 lines were actually somewhat higher than in the
7 control HLacZ cell line. Similar results were
8 obtained with the other drugs, as overexpression of
9 c-FLIP_L significantly decreased oxaliplatin- and
10 CPT-11-induced apoptosis, whereas c-FLIP_S
11 overexpression failed to inhibit chemotherapy-
12 induced apoptosis (Fig. 9B). The similar IC₅₀(72h)
13 doses observed in the c-FLIP_L-overexpressing cell
14 lines and the HLacZ cell line (Table 1) probably
15 reflects the fact that c-FLIP_L overexpression did
16 not affect chemotherapy-induced cell cycle arrest,
17 resulting in similar levels of growth inhibition
18 despite the differences in drug-induced apoptosis
19 observed in these cell lines.

20

21 **Example 4 Overexpression of c-FLIP_L inhibits**
22 **chemotherapy-induced Fas-mediated cell death.** To
23 further investigate the role of c-FLIP_L in
24 regulating Fas-mediated apoptosis following drug
25 treatment, we developed a panel of MCF-7 cell lines
26 overexpressing c-FLIP_L. We developed cell lines with
27 5-10-fold increased c-FLIP_L expression compared to
28 cells transfected with empty vector (Fig. 4A). The
29 c-FLIP_L -overexpressing cell lines FL44 and FL64 and
30 cells transfected with empty vector (EV68) were
31 taken forward for further characterisation. Cell
32 viability assays indicated that treatment of EV68

1 cells with 5-FU followed by CH-11 resulted in a
2 highly synergistic decrease in cell viability
3 (RI=2.06, $p<0.0005$) (Fig. 4B). However, no
4 synergistic decrease in cell viability was observed
5 in 5-FU and CH-11 co-treated FL44 or FL64 cells,
6 with RI values of 1.14 and 1.01 respectively (Fig.
7 4B). Furthermore, 5-FU and CH-11 co-treatment
8 resulted in caspase 8 activation and PARP cleavage
9 in EV68 cells (Fig. 4C). In contrast, c-FLIP_L
10 overexpression in FL64 cells abrogated both
11 activation of caspase 8 and PARP cleavage in
12 response to 5-FU and CH-11 co-treatment (Fig. 4C).
13
14 We next examined the effect of c-FLIP_L
15 overexpression on Fas-mediated apoptosis following
16 treatment with the antifolates TDX and MTA and the
17 DNA-damaging agent OXA. All three drugs
18 synergistically decreased cell viability in EV68
19 cells when combined with CH-11 (Figs. 5A and B).
20 However, this synergistic interaction was inhibited
21 by c-FLIP_L overexpression in both the FL44 and FL64
22 cell lines (Figs. 5A and B). Analysis of caspase 8
23 activation and PARP cleavage confirmed that Fas-
24 mediated apoptosis in response to all three agents
25 was attenuated by c-FLIP_L overexpression. Combined
26 treatment with each antifolate and CH-11 resulted in
27 caspase 8 activation in EV68 cells, but not FL64
28 cells (Fig. 5C). Similarly, PARP cleavage in
29 response to the antifolates and CH-11 was inhibited
30 in the FL64 cell line (Fig. 5C). Although some
31 caspase 8 activation and PARP cleavage were observed
32 in FL64 cells following co-treatment with 5 μ M OXA

1 and CH-11, this was much reduced compared to the
2 EV68 cell line (Fig. 5D). These results indicate
3 that c-FLIP_L is a key regulator of Fas-mediated
4 apoptosis in response to 5-FU, antifolates and
5 oxaliplatin.

6
7 Similar experiemnts were carried out using a number
8 of other cell lines and chemotherapeutic agents in
9 combination with CH-11. The results are shown in
10 Figure 9C. Treatment with 50ng/mL CH-11 in the
11 absence of chemotherapy induced a small degree of
12 apoptosis in the HLacZ control cell line (data not
13 shown). However, co-treatment with each chemotherapy
14 and CH-11 resulted in high levels of apoptosis in
15 the HLacZ cell line (Fig. 9C). High levels of
16 apoptosis were also observed in the c-FLIP_S-
17 overexpressing cell lines HFS19 and HFS44 in
18 response to chemotherapy and CH-11 (Fig. 9C). In
19 contrast, c-FLIP_L overexpression in the HFL17 and
20 HFL24 cell lines dramatically inhibited apoptosis in
21 response to co-treatment with each chemotherapy and
22 CH-11 (Fig. 9C). So, overexpression of c-FLIP_L, but
23 not c-FLIP_S, protected HCT116p53^{+/+} cells from both
24 chemotherapy-induced apoptosis and apoptosis induced
25 in response to co-treatment with chemotherapy and
26 the Fas agonist CH-11.

27
28 **Example 6 siRNA-targeting of c-FLIP sensitises**
29 **cancer cells to chemotherapy.**

30
31 Having established that c-FLIP_L overexpression
32 protected MCF-7 and HCT116 cells from chemotherapy-

1 induced Fas-mediated cell death, we next designed a
2 FLIP-targeted (FT) siRNA to inhibit both c-FLIP
3 splice variants. Transfection with 10nM FT siRNA
4 potently down-regulated expression of both c-FLIP
5 splice variants in MCF-7 cells (Fig. 6A). Cell
6 viability analysis of MCF-7 cells transfected with
7 FT siRNA indicated that co-treatment with 5-FU
8 resulted in a supra-additive decrease in cell
9 viability (Fig. 6B, $RI=1.56$, $p<0.005$).
10 Interestingly, transfection of MCF-7 cells with FT
11 siRNA significantly decreased cell viability in the
12 absence of co-treatment with 5-FU, with an
13 approximate 50% decrease in cell viability in cells
14 transfected with 2.5nM FT siRNA (Fig. 6B). A
15 scrambled control (SC) siRNA that had no effect of
16 FLIP expression, also had no effect on cell
17 viability either alone or in combination with 5-FU
18 (data not shown). The decrease in cell viability in
19 response to FT siRNA alone appeared to be due to the
20 induction of apoptosis, as transfection of FT siRNA
21 in the absence of co-treatment with drug induced
22 significant levels of PARP cleavage (Fig. 6C, lane
23 2). Furthermore, combined treatment with FT siRNA
24 and 5-FU resulted in potent cleavage of PARP (Fig.
25 6C), indicating that the synergistic decrease in
26 cell viability observed in MCF-7 cells co-treated
27 with these agents was due to increased apoptosis.
28
29 FT siRNA also potently down-regulated FLIP_L and FLIP_S
30 expression in HCT116 cells (Fig. 7A). Analysis of
31 caspase 8 activation in siRNA-transfected HCT116
32 cells indicated that FT siRNA alone (1nM) caused

1 some activation of caspase 8, as indicated by the
2 decrease in the levels of p53/55 zymogen and
3 appearance of the p41/43 cleavage products (Fig. 7B,
4 lane 3). This was accompanied by some PARP cleavage.
5 At higher concentrations ($>5\text{nM}$), FT siRNA alone
6 caused more potent activation of caspase 8 and PARP
7 cleavage in HCT116 cells (Fig. 7C). Both 5-FU ($5\mu\text{M}$)
8 and TDX (100nM) caused some caspase 8 activation in
9 mock and SC transfected HCT116 cells as indicated by
10 the presence of p41/p43 caspase 8, although no PARP
11 cleavage was observed in these cells (Fig. 7B). The
12 most potent activation of caspase 8 was observed in
13 cells co-treated with 1nM FT siRNA and 5-FU or TDX,
14 with decreased expression of the p53/55 zymogen and
15 increased expression of both the p41/43 and p18
16 caspase 8 cleavage products (Fig. 7B, lanes 6 and
17 9). Furthermore, activation of caspase 8 in FT
18 siRNA/chemotherapy-treated HCT116 cells was
19 accompanied by potent PARP cleavage. Cell viability
20 assays indicated that co-treatment with 0.5nM FT
21 siRNA and $5\mu\text{M}$ 5-FU resulted in a synergistic
22 decrease in cell viability (Fig. 8A, $\text{RI}=2.10$,
23 $p<0.0005$). In contrast, SC siRNA had no significant
24 effect on cell viability either in the presence or
25 absence of 5-FU. Furthermore, co-treatment with FT
26 siRNA and both TDX and OXA resulted in synergistic
27 decreases in cell viability, with RI values of 1.68
28 and 2.26 respectively (Figs. 8B and C). These
29 results indicate that inhibition of c-FLIP
30 expression in HCT116 and MCF-7 cells dramatically
31 sensitised them to chemotherapy-induced apoptosis.
32

1 Further evidence that siRNA-targeting of c-FLIP
2 sensitises HCT116p53^{+/+} cells to chemotherapy is
3 shown in Figure 11. FLIP-targeted siRNAs were
4 designed to down-regulate expression of both c-FLIP
5 splice variants. Of several siRNAs tested, one FLIP-
6 targeted (FT) siRNA potently down-regulated
7 expression of both c-FLIP splice variants in
8 HCT116p53^{+/+} cells at nanomolar concentrations (Fig.
9 11A). We used this FT siRNA to analyse the effect of
10 down-regulating c-FLIP expression on drug-induced
11 apoptosis. Interestingly, transfection with 0.5nM FT
12 siRNA in the absence of chemotherapy induced
13 significant levels of apoptosis (~26%) in
14 HCT116p53^{+/+} cells compared to cells transfected with
15 control siRNA (~9%) as assessed by flow cytometric
16 analysis of cells in the sub-G₀/G₁ apoptotic fraction
17 (p<0.0001; Fig. 11B). Importantly, co-treatment of
18 FT siRNA transfected cells with an IC₆₀_{72h} dose of 5-
19 FU for 48 hours resulted in a supra-additive
20 increase in apoptosis, with ~43% of cells undergoing
21 apoptosis compared to ~11% in 5-FU-treated cells
22 transfected with the control non-silencing siRNA
23 (p=0.0018; Fig. 11B). The results following
24 oxaliplatin treatment were even more dramatic, with
25 ~61 % of cells co-treated with FT siRNA and
26 oxaliplatin in the sub-G₁/G₀ phase after 48 hours,
27 compared to ~17% of cells co-treated with control
28 siRNA and oxaliplatin (p<0.0001; Fig. 11B).
29
30 Analysis of caspase 8 activation in siRNA-
31 transfected HCT116p53^{+/+} cells indicated that 0.5nM
32 FT siRNA alone caused some activation of caspase 8,

1 as indicated by the decrease in the levels of p53/55
2 zymogen and appearance of the p41/43 cleavage
3 products (Fig. 11C). Consistent with the cell cycle
4 data, transfection with 0.5nM FT siRNA resulted in
5 some PARP cleavage in the absence of chemotherapy.
6 Treatment with 5µM 5-FU also caused modest caspase 8
7 activation in mock-transfected cells and cells
8 transfected with control siRNA (as indicated by the
9 presence of p41/p43 caspase 8), however no PARP
10 cleavage was observed in these cells (Fig. 11C). By
11 far the most potent activation of caspase 8 was
12 observed in cells co-treated with 0.5nM FT siRNA and
13 5-FU, with decreased expression of the p53/55
14 zymogen and increased expression of the p41/43
15 caspase 8-cleavage product (Fig. 11C). Furthermore,
16 activation of caspase 8 in FT siRNA/5-FU-treated
17 HCT116p53^{+/+} cells was accompanied by complete PARP
18 cleavage. Similar results were obtained for the
19 antifolate tomudex, which is a specific inhibitor of
20 nucleotide synthetic enzyme thymidylate synthase (TS)
21 (Fig. 11C). Furthermore, potent caspase 8 activation
22 and PARP cleavage were observed in cells co-treated
23 with FT siRNA and oxaliplatin after 24 hours,
24 compared to cells treated with either agent
25 individually (Fig. 11C). In light of these results,
26 we also examined the effect of down-regulating c-
27 FLIP on apoptosis induced by CPT-11, another
28 chemotherapeutic drug currently used in the
29 treatment of colorectal cancer. As with the other
30 drugs, down-regulation of c-FLIP sensitised
31 HCT116p53^{+/+} cells to CPT-11-induced activation of
32 caspase 8 and apoptosis (Fig. 10C).

1
2 Given the more than additive effects of FT siRNA and
3 chemotherapy on apoptosis in HCT116p53^{+/+} cells, we
4 carried out cell viability assays to determine
5 whether the interactions were synergistic. Cell
6 viability assays indicated that co-treatment with FT
7 siRNA and 5-FU resulted in combination index (CI)
8 values of <1 for 8/9 concentrations (Fig. 11D).
9 According to the definitions of Chou and Talalay,
10 the CI values for FT siRNA/5-FU co-treatment
11 indicated that there was a moderate synergistic
12 interaction for 4/9 concentration combinations
13 examined and a synergistic interaction for a further
14 4 concentrations (Fig. 11D). Co-treatment with FT
15 siRNA and oxaliplatin resulted in synergistic
16 decreases in cell viability for all concentrations
17 examined, with CI values ranging from ~0.25-0.75
18 (Fig. 3D). Similarly, combined treatment with CPT-11
19 and FT siRNA resulted in synergistic or moderate
20 synergistic decreases in cell viability with CI
21 values ranging from ~0.50-0.85 (Fig. 11D). Control
22 siRNA had no effect on cell viability in the
23 presence or absence of any of the drugs (data not
24 shown). Collectively, these results indicate that
25 down-regulation of c-FLIP expression dramatically
26 sensitises HCT116p53^{+/+} cells to 5-FU-, oxaliplatin-
27 and CPT-11-induced apoptosis.

28

29 **Example 7A The agonistic Fas monoclonal antibody CH-**
30 **11 synergistically activates apoptosis in response**
31 **to CPT-11 and TDX in a p53-independent manner**

1 The agonistic anti-Fas antibody CH-11 has been shown
2 to activate the Fas/CD95 receptor and cause
3 apoptosis. Lack of up-regulation of the Fas/CD95
4 receptor in a p53 mutant colon cancer cell line
5 abolished the synergistic interaction between 5-FU
6 and CH-11. In our study treatment of the p53 wild-
7 type and null cell lines with a range of each of the
8 chemotherapy agents 5-FU, TDX, CPT-11 and
9 Oxaliplatin followed 24 hours later by the addition
10 of the anti-Fas antibody CH-11 (200ng/ml) for a
11 further 48 hours resulted in significant synergy for
12 all the drugs in the p53 wild-type setting, but in
13 the p53 null cells this synergy was only seen with
14 the topoisomerase-I inhibitor CPT-11 and the
15 thymidylate synthase inhibitor TDX. There was no
16 synergistic interaction seen at all with Oxaliplatin
17 in the p53 null cells at any dose, and only slight
18 interaction with 5-FU at the higher doses (Fig. 17).
19 Propidium iodide staining of the HCT116 p53 wild-
20 type and null cell lines treated with these
21 chemotherapeutic agents for 24 hours followed by CH-
22 11 50ng/ml for an additional 24 hours confirmed that
23 a synergistic interaction is seen with each of the
24 drugs and CH-11 in the p53 wild-type cells (Fig.
25 18), whereas in the p53 null setting only treatment
26 with CPT-11 and to a lesser extent with TDX resulted
27 in significant synergy with CH-11 50ng/ml.

28

29

30 **Example 7B Effect of p53 inactivation on the synergy**
31 **between CH-11 and 5-FU, CPT-11 and Oxaliplatin**

1 Activation of the Fas/CD95 receptor by its natural
2 ligand FasL or the monoclonal antibody CH-11 results
3 in the recruitment and activation of procaspase 8 at
4 the DISC. Procaspase 8 is cleaved to its active
5 subunits p41/43 and p18. Poly(ADP-ribose)polymerase
6 (PARP) is normally involved in DNA repair and
7 stability, and is cleaved by members of the caspase
8 family during early apoptosis.
9 Western blot analysis of the p53 wild-type and null
10 cell lines treated with IC60 doses of these
11 chemotherapeutic agents for 24 hours followed by a
12 further 24 hours of the anti-Fas antibody CH-11
13 (200ng/ml) resulted in PARP cleavage and activation
14 of procaspase 8 (with generation of the active
15 p41/43 and p18 subunits) in the p53 wild-type cell
16 line for each drug (Fig. 19). In the p53 null cell
17 line PARP cleavage and procaspase 8 activation
18 following the addition of CH-11 was only seen
19 following treatment with CPT-11.

20

21 **Example 7C Effect of p53 status on c-FLIP regulated**
22 **chemosensitivity**

23

24 In order to determine whether down-regulation of c-
25 FLIP would also sensitise p53 null HCT116 cells to
26 chemotherapy-induced apoptosis, we transfected these
27 cells with FT siRNA and co-treated them with
28 chemotherapy (5-FU, oxaliplatin and CPT-11). The p53
29 null cells (HCT116p53^{-/-}) expressed higher levels of
30 both c-FLIP splice forms than p53 wild type cells
31 (Fig. 12A), but expression was effectively down-
32 regulated by 1nM FT siRNA (Fig. 12B). Treatment of

1 the p53 null cells with 1nM FT siRNA alone resulted
2 in a modest increase in apoptosis after 72 hours,
3 with ~14% of cells in the sub-G₀/G₁ fraction compared
4 to ~9% in SC siRNA transfected cells (p=0.0081; Fig.
5 12C). Co-treatment of FT siRNA-transfected cells
6 with 5µM 5-FU significantly increased the apoptotic
7 fraction to ~29% compared to ~14% of 5-FU/SC siRNA
8 co-treated cells (p=0.0003; Fig. 12C). Treatment of
9 FT siRNA-transfected HCT116 p53 null cells with 5µM
10 oxaliplatin resulted in a highly significant
11 increase in cells undergoing apoptosis compared to
12 oxaliplatin/SC siRNA co-treated cells (~46% compared
13 to ~27%, p<0.0001; Fig. 4C). FT siRNA also increased
14 apoptosis of HCT116p53^{-/-} cells in response to 1µM
15 CPT-11 to ~33% compared to ~22% in SC/CPT-11 co-
16 treated cells (p=0.0002; Fig. 12C). These results
17 indicate that down-regulating c-FLIP expression
18 significantly enhanced chemotherapy-induced
19 apoptosis in p53 null HCT116 cells, in particular
20 oxaliplatin-induced apoptosis.

21
22 We further analysed the effect of down-regulating c-
23 FLIP on the chemosensitivity of p53 null HCT116
24 cells using the MTT cell viability assay. While
25 greater than additive increases in apoptosis were
26 detected for combined treatment with FT siRNA and 5-
27 FU in HCT116p53^{-/-} cells (Fig. 12C), cell viability
28 assays identified slight synergy in only 2/9
29 combinations (Fig. 12D). Similarly, the interaction
30 between FT siRNA and CPT-11 was found to be
31 moderately or slightly synergistic for only 3/9 drug
32 combinations (Fig. 12D). So, although c-FLIP down-

1 regulation sensitised HCT116p53^{-/-} cells to 5-FU-
2 and CPT-11-induced apoptosis (Fig. 12C), cell
3 viability assays indicated that fewer drug
4 combinations were synergistic than in the p53 wild
5 type parental cell line, and that the degree of
6 synergy was less. However, co-treatment of
7 HCT116p53^{-/-} cells with oxaliplatin and FT siRNA was
8 synergistic or moderately synergistic for all nine
9 combinations analysed, with CI values ranging from
10 ~0.35-0.85 (Fig. 12D), most likely reflecting the
11 greater level of apoptosis induced for this
12 combination than for the other chemotherapeutic
13 drugs (Fig. 12C).

14

15 **Effect of c-FLIP on chemosensitivity in other**
16 **colorectal cancer cell lines.** In order to determine
17 whether c-FLIP is a general modulator of
18 chemosensitivity in colorectal cancer, we extended
19 these studies into two further colorectal cancer
20 cell line models, namely the p53 wild type RKO cell
21 line and the p53 mutant H630 cell line. Each cell
22 line expressed both c-FLIP splice forms, and FT
23 siRNA down-regulated c-FLIP protein in both lines
24 (Fig. 13A). As in the HCT116 cell lines, down-
25 regulation of c-FLIP sensitised both cell lines to
26 apoptosis induced by 5-FU, oxaliplatin and CPT-11
27 (Fig. 5B). In each case, the effect of co-treatment
28 with chemotherapy and FT siRNA was more than
29 additive. Of note, the sensitisation to CPT-11 was
30 particularly marked in both lines, with ~43% of FT
31 siRNA/CPT-11 co-treated RKO cells undergoing
32 apoptosis compared to ~15% of SC siRNA/CPT-11 co-

1 treated RKO cells, and ~32% of FT siRNA/CPT-11 co-
2 treated H630 cells undergoing apoptosis compared to
3 ~12% of SC siRNA/CPT-11 co-treated H630 cells. MTT
4 analyses indicated synergistic interactions between
5 FT siRNA and each drug in RKO cells, with the
6 majority of CI values below 0.75 for each drug (Fig.
7 13C). The synergy was less pronounced in the H630
8 cells, with the combination of FT siRNA and CPT-11
9 being the most consistently synergistic or
10 moderately synergistic (Fig. 13C).

11

12 Collectively, these results indicate that c-FLIP
13 plays an important role in regulating chemotherapy-
14 induced apoptosis in colorectal cancer cell lines.
15 Furthermore, while both p53 wild type, mutant and
16 null cell lines are sensitised to chemotherapy-
17 induced apoptosis following down-regulation of c-
18 FLIP, the extent of synergy would appear to be less
19 in cell lines lacking functional p53.

20

21 **Potent knock-down of c-FLIP induces apoptosis in the**
22 **absence of chemotherapy.** As already discussed,
23 transfection of 0.5nM FT siRNA into HCT116p53^{+/+}
24 cells significantly increased apoptosis in the
25 absence of co-treatment with chemotherapy (Fig.
26 10B). When higher concentrations of FT siRNA were
27 used to more completely knock down expression of c-
28 FLIP in HCT116p53^{+/+} cells, a dramatic decrease in
29 cell viability (Fig. 14A) and a significant increase
30 in PARP cleavage and apoptosis was observed (Fig.
31 14B and C) in the absence of chemotherapy. A similar
32 effect was observed in HCT116p53^{-/-} cells, although

1 the extent of PARP cleavage and apoptosis was less
2 than in the p53 wild type cell line (Fig. 14B and
3 C). However, exposure of HCT116p53^{-/-} cells to higher
4 concentrations of FT siRNA for 72 hours resulted in
5 levels of apoptosis that approached those observed
6 in the p53 wild type parental cell line (Fig. 14D).
7 The IC_{50(72h)} doses of FT siRNA in the p53 wild type
8 and null cell lines were ~0.7nM and ~2.5nM
9 respectively as determined by MTT assay. FT siRNA
10 also potently induced apoptosis in RKO and H630
11 cells in the absence of chemotherapy (Fig. 14E and
12 F). The IC_{50(72h)} doses in these cell lines were
13 calculated to be ~5nM in RKO cells and ~25nM in H630
14 cells. These results indicate that c-FLIP may be a
15 general determinant of colorectal cancer cell
16 viability even in the absence of cytotoxic drugs.
17 Furthermore, targeting c-FLIP induced apoptosis in
18 p53 wild type, mutant and null and colorectal cancer
19 cells, suggesting that it may represent an important
20 new therapeutic target for treating this disease.

21
22 Examination of the kinetics of c-FLIP down-
23 regulation following FT siRNA transfection indicated
24 that both splice forms were efficiently down-
25 regulated as early as 8 hours post-transfection
26 (Fig. 15A). This is in agreement with previous
27 findings, which indicate that c-FLIP is rapidly
28 turned over in cells following treatment with the
29 protein synthesis inhibitor cycloheximide (16).
30 Down-regulation of c-FLIP at 8 hours correlated with
31 decreased levels of procaspase 8 and the onset of
32 apoptosis as indicated by PARP cleavage (Fig. 15A).

1 This was more apparent for the higher concentration
2 of FT siRNA (10nM). By 12 and 24 hours post-
3 transfection, the p41/43-caspase 8 cleavage
4 fragments could be detected in addition to the
5 decrease in procaspase 8 levels and PARP cleavage in
6 response to 1nM and 10nM FT siRNA (Fig. 15A). In
7 agreement with the Western blot analysis, flow
8 cytometry indicated that the onset of apoptosis
9 following FT siRNA transfection occurred between 6
10 and 12 hours (Fig. 15B). Therefore, c-FLIP down-
11 regulation would appear to be tightly coupled to
12 caspase 8 activation and the onset of apoptosis.

13

14 **Effect of specific targeting of c-FLIP_L on**
15 **apoptosis.** Our initial observation was that
16 activation of apoptosis in chemotherapy/CH-11-
17 treated HCT116p53^{+/+} cells coincided with loss of
18 full-length c-FLIP_L (Fig. 9B). It was therefore
19 possible that the effects on cell survival of down-
20 regulating both c-FLIP splice variants were actually
21 a result of the down-regulation of c-FLIP_L. In
22 addition, data from the c-FLIP overexpressing cell
23 lines suggested that c-FLIP_L was the more important
24 regulator of chemoresistance (Fig. 10B). So, we
25 designed an siRNA to specifically down-regulate the
26 long splice form without affecting expression of c-
27 FLIP_S (Fig. 16A). Similar to the effect of the dual-
28 targeted siRNA, specific down-regulation of c-FLIP_L
29 induced apoptosis of HCT116p53^{+/+} cells in the
30 absence of chemotherapy, as indicated by PARP
31 cleavage (Fig. 8A) and flow cytometry (data not
32 shown). Furthermore, combined treatment with FL

1 siRNA and each chemotherapy resulted in enhanced
2 apoptosis (Fig. 16B) and highly synergistic
3 decreases in cell viability (Fig. 16C). Similar
4 synergistic decreases in cell viability were
5 observed in the H630 and RKO cell lines (data not
6 shown). These data suggest that down-regulation of
7 c-FLIP_L is sufficient to recapitulate the effects of
8 down-regulating both splice variants and that, of
9 the two splice forms, c-FLIP_L may be the more
10 critical regulator of colorectal cancer cell death.

11

12

13 DISCUSSION

14

15 We found that the Fas death receptor was highly up-
16 regulated in response to 5-FU, the TS-targeted
17 antifolates TDX and MTA and the DNA-damaging agent
18 OXA in MCF-7 breast cancer and HCT116 colon cancer
19 cells, however, this did not result in significant
20 activation of apoptosis. Expression of FasL by
21 activated T cells and natural killer cells induces
22 apoptosis of Fas expressing target cells in vivo
23 (O'Connell et al., 1999). To mimic the effects of
24 these immune effector cells in our in vitro model,
25 we used the agonistic Fas monoclonal antibody CH-11.
26 We found that CH-11 potently activated apoptosis of
27 chemotherapy-treated cells, suggesting that the Fas
28 signalling pathway is an important mediator of
29 apoptosis in response to these agents in vivo. Many
30 tumour cells overexpress FasL, and it has been
31 postulated that tumour FasL induces apoptosis of
32 Fas-sensitive immune effector cells, thereby

1 inhibiting the antitumor immune response (O'Connell
2 et al., 1999). This hypothesis has been supported by
3 both in vitro and in vivo studies (Bennett et al.,
4 1998; O'Connell et al., 1997). The strategy of
5 overexpressing FasL requires that the tumour cells
6 develop resistance to Fas-mediated apoptosis to
7 prevent autocrine and paracrine induction of tumour
8 cell death. The lack of caspase 8 activation that we
9 observed in response to chemotherapy suggests that
10 Fas-mediated apoptosis may be inhibited in MCF-7 and
11 HCT116 and cancer cells, but that co-treatment with
12 CH-11 was sufficient to overcome this resistance and
13 activate Fas-mediated apoptosis.

14
15 Fas signalling may be inhibited by c-FLIP, which can
16 inhibit caspase 8 recruitment to and activation at
17 the Fas DISC (Krueger et al., 2001). Multiple c-FLIP
18 splice variants have been reported, however, only
19 two forms (c-FLIP_L and c-FLIP_S) have been detected at
20 the protein level (Scaffidi et al., 1999). Both
21 splice variants have death effector domains (DEDs),
22 with which they bind to FADD, blocking access of
23 procaspase 8 molecules to the DISC. c-FLIP_L is
24 processed at the DISC as it is a natural substrate
25 for caspase 8, which cleaves it to generate a
26 truncated form of approximately 43kDa (p43-FLIPL)
27 (Niikura et al., 2002). Cleaved p43- c-FLIP_L binds
28 more tightly to the DISC than full-length c-FLIP_L.
29 c-FLIP_S is not processed by caspase 8 at the DISC.
30 c-FLIP_L appears to be a more potent inhibitor of
31 Fas-mediated cell death than c-FLIP_S (Irmeler et al.,
32 1997; Tschopp et al., 1998). Initially both pro-

1 apoptotic and anti-apoptotic effects were proposed
2 for c-FLIP. However, enhanced cell death occurred
3 mainly in experiments using transient over-
4 expression and may have been due to excessive levels
5 of these DED-containing proteins, which may have
6 caused clustering of other DED-containing proteins
7 including procaspase 8, resulting in caspase
8 activation (Siegel et al., 1998). The data from cell
9 lines stably over-expressing c-FLIP and from mice
10 deficient in c-FLIP support an anti-apoptotic
11 function for c-FLIP (Yeh et al., 2000).

12

13 We found that c-FLIP_L was up-regulated and processed
14 to its p43-form in MCF-7 cells following treatment
15 with 5-FU and TDX. Furthermore, activation of
16 caspase 8 and apoptosis in cells co-treated with
17 chemotherapy and CH-11 coincided with processing of
18 c-FLIP_L. These results suggested that c-FLIP_L
19 regulated the onset of drug-induced Fas-mediated
20 apoptosis in these cell lines. This hypothesis was
21 further supported by data from overexpression and
22 siRNA studies. c-FLIP overexpression abrogated the
23 synergistic interaction between CH-11 and 5-FU, TDX,
24 MTA and OXA by inhibiting caspase 8 activation.
25 Furthermore, siRNA-targeting of both c-FLIP splice
26 variants sensitised cells to these chemotherapeutic
27 agents as determined by cell viability and PARP
28 cleavage assays. Collectively, these results
29 indicate that c-FLIP inhibits apoptosis in response
30 to these drugs.

31

1 Surprisingly, we also found that siRNA-mediated
2 down-regulation of c-FLIP_L and c-FLIP_S induced
3 caspase 8 activation and PARP cleavage in the
4 absence of co-treatment with chemotherapy (although
5 co-treatment with drug enhanced the effect). The
6 inventors found that overexpression of c-FLIP_L
7 protected HCT116 cells from chemotherapy-induced
8 apoptosis and apoptosis induced following co-
9 treatment with chemotherapy and the Fas agonistic
10 antibody CH-11. In addition to blocking caspase 8
11 activation, DISC-bound c-FLIP has been reported to
12 promote activation of the ERK, PI3-kinase/Akt and
13 NFκB signalling pathways (Kataoka et al., 2000;
14 Panka et al., 2001). The NFκB, PI3K/Akt and ERK
15 signal transduction pathways are associated with
16 cell survival and/or proliferation, therefore, c-
17 FLIP is capable of both blocking caspase 8
18 activation and also recruiting adaptor proteins that
19 can activate intrinsic survival and proliferation
20 pathways (Shu et al., 1997). Furthermore, c-FLIP
21 also inhibits procaspase 8 activation at the DISCs
22 formed by the TRAIL receptors DR4 and DR5 (Krueger
23 et al., 2001). rTRAIL induces apoptosis in a range
24 of human cancer cell lines including colorectal and
25 breast, indicating that the TRAIL receptors are
26 widely expressed in tumour cells (Ashkenazi, 2002).
27 It is possible that expression of DR4 and DR5 is
28 tolerated in tumours because c-FLIP converts the
29 apoptotic signal to one which promotes survival and
30 proliferation. Thus, siRNA-mediated down-regulation
31 of c-FLIP may induce apoptosis by inhibiting FLIP-

1 mediated activation of NF κ B, PI3K/Akt and ERK and
2 promoting activation of caspase 8 at TRAIL DISCs.

3

4 We have found that c-FLIP is a key regulator of Fas-
5 mediated apoptosis in response to 5-FU, TS-targeted
6 antifolates and OXA. Our results suggest that c-FLIP
7 may be a clinically useful predictive marker of
8 response to these agents and that c-FLIP is a
9 therapeutically attractive target.

10

11 Furthermore, Our findings indicate that c-FLIP_L
12 overexpression inhibits apoptosis of colorectal
13 cancer cells in response to the chemotherapeutic
14 agents used in the treatment of colorectal cancer
15 (5-FU, oxaliplatin and CPT-11). This has particular
16 clinical relevance given the high incidence of c-
17 FLIP_L overexpression observed in colorectal cancer
18 (6) and suggests that c-FLIP_L overexpression may
19 contribute to chemoresistance in colorectal cancer.
20 Interestingly, c-FLIP_S overexpression failed to
21 protect colorectal cancer cells from chemotherapy-
22 induced apoptosis, or apoptosis induced by co-
23 treatment with chemotherapy and CH-11. These results
24 would suggest that, of the two splice forms, c-FLIP_L
25 is the more important mediator of resistance to
26 chemotherapy in colorectal cancer cells.

27

28 Our study indicates that down-regulating c-FLIP in a
29 panel of colorectal cancer cells that have not been
30 selected for drug resistance increases their
31 sensitivity to a range of cytotoxic drugs with
32 differing mechanisms of action. Furthermore, the

1 study has demonstrated that the down-regulation of
2 c-FLIP alone can induce apoptosis .

3
4 It would appear from our c-FLIP overexpressing cell
5 lines and studies using a c-FLIP_L-specific siRNA
6 that the long splice form may be the more important
7 in mediating survival of colorectal cancer cells,
8 however conclusive proof of this will require the
9 generation of a c-FLIP_S-specific siRNA. The
10 induction of apoptosis following c-FLIP knock-down
11 is most likely mediated by death receptors such as
12 Fas and DR5. We have previously shown that Fas is
13 up-regulated in response to 5-FU in HCT116p53^{+/+} and
14 RKO cells, but not in HCT116p53^{-/-} and H630 cells
15 (39), while DR5 is constitutively expressed in both
16 HCT116 cell lines and the RKO and H630 lines
17 (unpublished observations). It is possible that
18 knocking down c-FLIP expression (either in the
19 presence or absence of chemotherapy) removes c-FLIP-
20 mediated inhibition of caspase 8 activation at Fas
21 and/or DR5 DISCs, leading to caspase 8-mediated
22 activation of apoptosis. Indeed, our initial
23 evidence suggests that the onset of apoptosis and
24 caspase 8 activation following c-FLIP knock-down are
25 tightly coupled. In addition to blocking caspase 8
26 activation, DISC-bound c-FLIP has been reported to
27 promote activation of the anti-apoptotic ERK, PI3-
28 kinase/Akt and NF- κ B signalling pathways (7, 8). So,
29 it is also possible that loss of c-FLIP eliminates
30 DISC-dependent up-regulation of these survival
31 pathways, leading to enhanced susceptibility to
32 apoptosis. In addition, a recent study has suggested

1 that c-FLIP_L may have a non-DISC-dependent anti-
2 apoptotic function by binding to and inhibiting pro-
3 apoptotic signalling via p38 MAPK (40).
4

5 The p53 tumour suppressor gene is mutated in 40-60%
6 of colorectal cancers most often in the central DNA-
7 binding core domain responsible for sequence-
8 specific binding to transcriptional target genes
9 (41). p53 has been reported to both
10 transcriptionally up-regulate c-FLIP (42) and target
11 it for ubiquitin-mediated degradation by the
12 proteasome (43), suggesting that the effect of p53
13 on c-FLIP expression is complex. In the present
14 study, we consistently found that expression of both
15 c-FLIP splice forms was higher in the p53 null
16 HCT116 cell line compared to the isogenic p53 wild
17 type line. We also examined how p53 status affected
18 cell viability when c-FLIP was down-regulated.
19 Although siRNA targeting of c-FLIP significantly
20 enhanced chemotherapy-induced apoptosis in p53 null
21 HCT116 cells, the effect was not as dramatic as in
22 the p53 wild type line. Similarly, the induction of
23 apoptosis after a 48 hour exposure to FLIP-targeted
24 siRNA alone was greater in the p53 wild type
25 setting. However, longer exposure times (72 hours)
26 and higher concentrations (10-100nM) of FT siRNA
27 induced levels of apoptosis in the HCT116 p53 null
28 cell line that approached those observed in the p53
29 wild type parental cell line. It is possible that
30 the differential sensitivity of the p53 wild type
31 and null cells to FT siRNA was at least partly due
32 to the higher constitutive levels of c-FLIP

1 expression in the p53 null line. It may also reflect
2 lower levels of basal and chemotherapy-induced
3 expression of the p53-regulated genes encoding the
4 Fas and DR5 death receptors in the p53 null cell
5 line, which lowers its sensitivity to loss of c-FLIP
6 expression. Of note, down-regulation of c-FLIP
7 markedly enhanced apoptosis in response to
8 oxaliplatin in the p53 null cells, which are usually
9 highly resistant to oxaliplatin (15). Further
10 analyses revealed that the effects of targeting c-
11 FLIP on chemotherapy-induced apoptosis were not
12 confined to the HCT116 lines, as similar results
13 were obtained in the p53 wild type RKO and p53
14 mutant H630 lines. Moreover, more potent knock down
15 of c-FLIP with higher concentrations of siRNA
16 triggered apoptosis in the absence of chemotherapy
17 in both RKO and H630 cell lines. Collectively these
18 results suggest that c-FLIP is an important
19 regulator of cell survival in p53 wild type, null
20 and mutant colorectal cancer cells in the presence
21 and absence of chemotherapy.

22
23 These findings have direct clinical relevance as 5-
24 FU/leucovorin/oxaliplatin (FOLFOX) and 5-
25 FU/leucovorin/CPT-11 (FOLFIRI) combination
26 chemotherapies are currently widely used in the
27 treatment of advanced colorectal cancer, and FOLFOX
28 has recently been demonstrated to improve 3-year
29 survival compared to 5-FU/leucovorin in the adjuvant
30 setting of the disease (78.2% versus 72.9%, $p=0.002$)
31 (44). Furthermore, clinical studies have
32 demonstrated significantly elevated c-FLIP

1 expression in colorectal and gastric tumours (6,
2 45), suggesting that c-FLIP may not only be a
3 relevant clinical target in colorectal cancer, but
4 also in gastric cancer, where 5-FU-based
5 chemotherapy regimens are also used. In conclusion,
6 this study suggests that c-FLIP may represent an
7 important clinical marker of drug resistance in
8 colorectal cancer and that targeting c-FLIP, either
9 alone, or in combination with standard
10 chemotherapies has therapeutic potential for the
11 treatment of this disease.

12

1
2
3 All documents referred to in this specification are
4 herein incorporated by reference. Various
5 modifications and variations to the described
6 embodiments of the inventions will be apparent to
7 those skilled in the art without departing from the
8 scope and spirit of the invention. Although the
9 invention has been described in connection with
10 specific preferred embodiments, it should be
11 understood that the invention as claimed should not
12 be unduly limited to such specific embodiments.
13 Indeed, various modifications of the described modes
14 of carrying out the invention which are obvious to
15 those skilled in the art are intended to be covered
16 by the present invention.

17

18 REFERENCES

19 Aherne, G. W., Hardcastle, A., Raynaud, F., and
20 Jackman, A. L. (1996). Immunoreactive dUMP and TTP
21 pools as an index of thymidylate synthase
22 inhibition; effect of tomudex (ZD1694) and a
23 nonpolyglutamated quinazoline antifolate (CB30900)
24 in L1210 mouse leukaemia cells. Biochem Pharmacol
25 51, 1293-1301.
26 Ashkenazi, A. (2002). Targeting death and decoy
27 receptors of the tumour-necrosis factor superfamily.
28 Nat Rev Cancer 2, 420-430.
29 Bennett, M. W., O'Connell, J., O'Sullivan, G. C.,
30 Brady, C., Roche, D., Collins, J. K., and Shanahan,
31 F. (1998). The Fas counterattack in vivo: apoptotic
32 depletion of tumor-infiltrating lymphocytes

1 associated with Fas ligand expression by human
2 esophageal carcinoma. J Immunol 160, 5669-5675.
3 Giacchetti, S., Perpoint, B., Zidani, R., Le Bail,
4 N., Faggiuolo, R., Focan, C., Chollet, P., Llory, J.
5 F., Letourneau, Y., Coudert, B., et al. (2000).
6 Phase III multicenter randomized trial of
7 oxaliplatin added to chronomodulated fluorouracil-
8 leucovorin as first-line treatment of metastatic
9 colorectal cancer. J Clin Oncol 18, 136-147.
10 Hughes, L. R., Stephens, T. C., Boyle, F. T., and
11 Jackman, A. L. (1999). Raltitrexed (TomudexTM), a
12 highly polyglutamatable antifolate thymidylate
13 synthase inhibitor, Humana press).
14 Irmeler, M., Thome, M., Hahne, M., Schneider, P.,
15 Hofmann, K., Steiner, V., Bodmer, J. L., Schroter,
16 M., Burns, K., Mattmann, C., et al. (1997).
17 Inhibition of death receptor signals by cellular
18 FLIP. Nature 388, 190-195.
19 Kataoka, T., Budd, R. C., Holler, N., Thome, M.,
20 Martinon, F., Irmeler, M., Burns, K., Hahne, M.,
21 Kennedy, N., Kovacsovics, M., and Tschopp, J.
22 (2000). The caspase-8 inhibitor FLIP promotes
23 activation of NF-kappaB and Erk signaling pathways.
24 Curr Biol 10, 640-648.
25 Kern, D. H., Morgan, C. R., and Hildebrand-Zanki, S.
26 U. (1988). In vitro pharmacodynamics of 1-beta-D-
27 arabinofuranosylcytosine: synergy of antitumor
28 activity with cis-diamminedichloroplatinum(II).
29 Cancer Res 48, 117-121.
30 Krueger, A., Baumann, S., Krammer, P. H., and
31 Kirchhoff, S. (2001). FLICE-inhibitory proteins:

- 1 regulators of death receptor-mediated apoptosis. *Mol*
2 *Cell Biol* 21, 8247-8254.
- 3 Longley, D. B., Boyer, J., Allen, W. L., Latif, T.,
4 Ferguson, P. R., Maxwell, P. J., McDermott, U.,
5 Lynch, M., Harkin, D. P., and Johnston, P. G.
6 (2002). The role of thymidylate synthase induction
7 in modulating p53-regulated gene expression in
8 response to 5-fluorouracil and antifolates. *Cancer*
9 *Res* 62, 2644-2649.
- 10 Longley, D. B., Ferguson, P. R., Boyer, J., Latif,
11 T., Lynch, M., Maxwell, P., Harkin, D. P., and
12 Johnston, P. G. (2001). Characterization of a
13 thymidylate synthase (TS)-inducible cell line: a
14 model system for studying sensitivity to TS- and
15 non-TS-targeted chemotherapies. *Clin Cancer Res* 7,
16 3533-3539.
- 17 Longley, D. B., Harkin, D. P., and Johnston, P. G.
18 (2003). 5-fluorouracil: mechanisms of action and
19 clinical strategies. *Nat Rev Cancer* 3, 330-338.
- 20 Mosmann, T. (1983). Rapid colorimetric assay for
21 cellular growth and survival: application to
22 proliferation and cytotoxicity assays. *J Immunol*
23 *Methods* 65, 55-63.
- 24 Nagata, S. (1999). Fas ligand-induced apoptosis.
25 *Annu Rev Genet* 33, 29-55.
- 26 Niikura, Y., Nonaka, T., and Imajoh-Ohmi, S. (2002).
27 Monitoring of caspase-8/FLICE processing and
28 activation upon Fas stimulation with novel
29 antibodies directed against a cleavage site for
30 caspase-8 and its substrate, FLICE-like inhibitory
31 protein (FLIP). *J Biochem (Tokyo)* 132, 53-62.

- 1 O'Connell, J., Bennett, M. W., O'Sullivan, G. C.,
2 Collins, J. K., and Shanahan, F. (1997). The Fas
3 counterattack: a molecular mechanism of tumor immune
4 privilege. *Mol Med* 3, 294-300.
- 5 O'Connell, J., Bennett, M. W., O'Sullivan, G. C.,
6 Collins, J. K., and Shanahan, F. (1999). Resistance
7 to Fas (APO-1/CD95)-mediated apoptosis and
8 expression of Fas ligand in esophageal cancer: the
9 Fas counterattack. *Dis Esophagus* 12, 83-89.
- 10 Panka, D. J., Mano, T., Suhara, T., Walsh, K., and
11 Mier, J. W. (2001). Phosphatidylinositol 3-
12 kinase/Akt activity regulates c-FLIP expression in
13 tumor cells. *J Biol Chem* 276, 6893-6896.
- 14 Romanelli, S., Perego, P., Pratesi, G., Carenini,
15 N., Tortoreto, M., and Zunino, F. (1998). In vitro
16 and in vivo interaction between cisplatin and
17 topotecan in ovarian carcinoma systems. *Cancer*
18 *Chemother Pharmacol* 41, 385-390.
- 19 Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W., and
20 Park, J. H. (2001). Increased expression of cFLIP(L)
21 in colonic adenocarcinoma. *J Pathol* 194, 15-19.
- 22 Scaffidi, C., Schmitz, I., Krammer, P. H., and
23 Peter, M. E. (1999). The role of c-FLIP in
24 modulation of CD95-induced apoptosis. *J Biol Chem*
25 274, 1541-1548.
- 26 Shih, C., Chen, V. J., Gossett, L. S., Gates, S. B.,
27 MacKellar, W. C., Habeck, L. L., Shackelford, K. A.,
28 Mendelsohn, L. G., Soose, D. J., Patel, V. F., et
29 al. (1997). LY231514, a pyrrolo[2,3-d]pyrimidine-
30 based antifolate that inhibits multiple folate-
31 requiring enzymes. *Cancer Res* 57, 1116-1123.

- 1 Shu, H. B., Halpin, D. R., and Goeddel, D. V.
- 2 (1997). Casper is a FADD- and caspase-related
- 3 inducer of apoptosis. *Immunity* 6, 751-763.
- 4 Siegel, R. M., Martin, D. A., Zheng, L., Ng, S. Y.,
- 5 Bertin, J., Cohen, J., and Lenardo, M. J. (1998).
- 6 Death-effector filaments: novel cytoplasmic
- 7 structures that recruit caspases and trigger
- 8 apoptosis. *J Cell Biol* 141, 1243-1253.
- 9 Tschopp, J., Irmeler, M., and Thome, M. (1998).
- 10 Inhibition of fas death signals by FLIPs. *Curr Opin*
- 11 *Immunol* 10, 552-558.
- 12 Yeh, W. C., Itie, A., Elia, A. J., Ng, M., Shu, H.
- 13 B., Wakeham, A., Mirtsos, C., Suzuki, N., Bonnard,
- 14 M., Goeddel, D. V., and Mak, T. W. (2000).
- 15 Requirement for Casper (c-FLIP) in regulation of
- 16 death receptor-induced apoptosis and embryonic
- 17 development. *Immunity* 12, 633-642.
- 18 Aherne, G. W., Hardcastle, A., Raynaud, F., and
- 19 Jackman, A. L. (1996). Immunoreactive dUMP and TTP
- 20 pools as an index of thymidylate synthase
- 21 inhibition; effect of tomudex (ZD1694) and a
- 22 nonpolyglutamated quinazoline antifolate (CB30900)
- 23 in L1210 mouse leukaemia cells. *Biochem Pharmacol*
- 24 51, 1293-1301.
- 25 Bennett, M., Macdonald, K., Chan, S. W., Luzio, J.
- 26 P., Simari, R., and Weissberg, P. (1998a). Cell
- 27 surface trafficking of Fas: a rapid mechanism of
- 28 p53-mediated apoptosis. *Science* 282, 290-293.
- 29 Bennett, M. W., O'Connell, J., O'Sullivan, G. C.,
- 30 Brady, C., Roche, D., Collins, J. K., and Shanahan,
- 31 F. (1998b). The Fas counterattack in vivo: apoptotic
- 32 depletion of tumor-infiltrating lymphocytes

1 associated with Fas ligand expression by human
2 esophageal carcinoma. J Immunol 160, 5669-5675.
3 Bunz, F., Hwang, P. M., Torrance, C., Waldman, T.,
4 Zhang, Y., Dillehay, L., Williams, J., Lengauer, C.,
5 Kinzler, K. W., and Vogelstein, B. (1999).
6 Disruption of p53 in human cancer cells alters the
7 responses to therapeutic agents. J Clin Invest 104,
8 263-269.
9 Chou, T. C., and Talalay, P. (1984). Quantitative
10 analysis of dose-effect relationships: the combined
11 effects of multiple drugs or enzyme inhibitors. Adv
12 Enzyme Regul 22, 27-55.
13 Chu, E., Koeller, D. M., Johnston, P. G., Zinn, S.,
14 and Allegra, C. J. (1993). Regulation of thymidylate
15 synthase in human colon cancer cells treated with 5-
16 fluorouracil and interferon-gamma. Mol Pharmacol 43,
17 527-533.
18 Giacchetti, S., Perpoint, B., Zidani, R., Le Bail,
19 N., Faggiuolo, R., Focan, C., Chollet, P., Llory, J.
20 F., Letourneau, Y., Coudert, B., et al. (2000).
21 Phase III multicenter randomized trial of
22 oxaliplatin added to chronomodulated fluorouracil-
23 leucovorin as first-line treatment of metastatic
24 colorectal cancer. J Clin Oncol 18, 136-147.
25 Hughes, L. R., Stephens, T. C., Boyle, F. T., and
26 Jackman, A. L. (1999). Raltitrexed (TomudexTM), a
27 highly polyglutamatable antifolate thymidylate
28 synthase inhibitor, Humana press).
29 Ichikawa, K., Yoshida-Kato, H., Ohtsuki, M., Ohsumi,
30 J., Yamaguchi, J., Takahashi, S., Tani, Y.,
31 Watanabe, M., Shiraishi, A., Nishioka, K., et al.
32 (2000). A novel murine anti-human Fas mAb which

- 1 mitigates lymphadenopathy without hepatotoxicity.
2 Int Immunol 12, 555-562.
- 3 Jiang, S., Song, M. J., Shin, E. C., Lee, M. O.,
4 Kim, S. J., and Park, J. H. (1999). Apoptosis in
5 human hepatoma cell lines by chemotherapeutic drugs
6 via Fas-dependent and Fas-independent pathways.
7 Hepatology 29, 101-110.
- 8 Kano, Y., Ohnuma, T., Okano, T., and Holland, J. F.
9 (1988). Effects of vincristine in combination with
10 methotrexate and other antitumor agents in human
11 acute lymphoblastic leukemia cells in culture.
12 Cancer Res 48, 351-356.
- 13 Kern, D. H., Morgan, C. R., and Hildebrand-Zanki, S.
14 U. (1988). In vitro pharmacodynamics of 1-beta-D-
15 arabinofuranosylcytosine: synergy of antitumor
16 activity with cis-diamminedichloroplatinum(II).
17 Cancer Res 48, 117-121.
- 18 Krueger, A., Baumann, S., Krammer, P. H., and
19 Kirchhoff, S. (2001). FLICE-inhibitory proteins:
20 regulators of death receptor-mediated apoptosis. Mol
21 Cell Biol 21, 8247-8254.
- 22 Longley, D. B., Boyer, J., Allen, W. L., Latif, T.,
23 Ferguson, P. R., Maxwell, P. J., McDermott, U.,
24 Lynch, M., Harkin, D. P., and Johnston, P. G.
25 (2002a). The role of thymidylate synthase induction
26 in modulating p53-regulated gene expression in
27 response to 5-fluorouracil and antifolates. Cancer
28 Res 62, 2644-2649.
- 29 Longley, D. B., Ferguson, P. R., Boyer, J., Latif,
30 T., Lynch, M., Maxwell, P., Harkin, D. P., and
31 Johnston, P. G. (2001). Characterization of a
32 thymidylate synthase (TS)-inducible cell line: a

- 1 model system for studying sensitivity to TS- and
2 non-TS-targeted chemotherapies. Clin Cancer Res 7,
3 3533-3539.
- 4 Longley, D. B., Harkin, D. P., and Johnston, P. G.
5 (2003). 5-fluorouracil: mechanisms of action and
6 clinical strategies. Nat Rev Cancer 3, 330-338.
- 7 Longley, D. B., McDermott, U., and Johnston, P. G.
8 (2002b). Clinical significance of prognostic and
9 predictive markers in colorectal cancer.
10 Pharmacogenomics J 2, 209-216.
- 11 Mosmann, T. (1983). Rapid colorimetric assay for
12 cellular growth and survival: application to
13 proliferation and cytotoxicity assays. J Immunol
14 Methods 65, 55-63.
- 15 Muller, M., Wilder, S., Bannasch, D., Israeli, D.,
16 Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle,
17 P. R., Stremmel, W., Oren, M., and Krammer, P. H.
18 (1998). p53 activates the CD95 (APO-1/Fas) gene in
19 response to DNA damage by anticancer drugs. J Exp
20 Med 188, 2033-2045.
- 21 Munsch, D., Watanabe-Fukunaga, R., Bourdon, J. C.,
22 Nagata, S., May, E., Yonish-Rouach, E., and
23 Reisdorf, P. (2000). Human and mouse Fas (APO-
24 1/CD95) death receptor genes each contain a p53-
25 responsive element that is activated by p53 mutants
26 unable to induce apoptosis. J Biol Chem 275, 3867-
27 3872.
- 28 Nagata, S. (1999). Fas ligand-induced apoptosis.
29 Annu Rev Genet 33, 29-55.
- 30 O'Connell, J., Bennett, M. W., O'Sullivan, G. C.,
31 Collins, J. K., and Shanahan, F. (1997). The Fas

1 counterattack: a molecular mechanism of tumor immune
2 privilege. *Mol Med* 3, 294-300.

3 O'Connell, J., Bennett, M. W., O'Sullivan, G. C.,
4 Collins, J. K., and Shanahan, F. (1999). Resistance
5 to Fas (APO-1/CD95)-mediated apoptosis and
6 expression of Fas ligand in esophageal cancer: the
7 Fas counterattack. *Dis Esophagus* 12, 83-89.

8 Pitti, R. M., Marsters, S. A., Lawrence, D. A., Roy,
9 M., Kischkel, F. C., Dowd, P., Huang, A., Donahue,
10 C. J., Sherwood, S. W., Baldwin, D. T., et al.
11 (1998). Genomic amplification of a decoy receptor
12 for Fas ligand in lung and colon cancer. *Nature* 396,
13 699-703.

14 Romanelli, S., Perego, P., Pratesi, G., Carenini,
15 N., Tortoreto, M., and Zunino, F. (1998). In vitro
16 and in vivo interaction between cisplatin and
17 topotecan in ovarian carcinoma systems. *Cancer*
18 *Chemother Pharmacol* 41, 385-390.

19 Ruberti, G., Cascino, I., Papoff, G., and Eramo, A.
20 (1996). Fas splicing variants and their effect on
21 apoptosis. *Adv Exp Med Biol* 406, 125-134.

22 Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W., and
23 Park, J. H. (2001). Increased expression of cFLIP(L)
24 in colonic adenocarcinoma. *J Pathol* 194, 15-19.

25 Sato, T., Irie, S., Kitada, S., and Reed, J. C.
26 (1995). FAP-1: a protein tyrosine phosphatase that
27 associates with Fas. *Science* 268, 411-415.

28 Shih, C., Chen, V. J., Gossett, L. S., Gates, S. B.,
29 MacKellar, W. C., Habeck, L. L., Shackelford, K. A.,
30 Mendelsohn, L. G., Soose, D. J., Patel, V. F., et
31 al. (1997). LY231514, a pyrrolo[2,3-d]pyrimidine-

- 1 based antifolate that inhibits multiple folate-
2 requiring enzymes. *Cancer Res* 57, 1116-1123.
- 3 Swain, S. M., Lippman, M. E., Egan, E. F., Drake, J.
4 C., Steinberg, S. M., and Allegra, C. J. (1989).
5 Fluorouracil and high-dose leucovorin in previously
6 treated patients with metastatic breast cancer. *J*
7 *Clin Oncol* 7, 890-899.
- 8 Tillman, D. M., Petak, I., and Houghton, J. A.
9 (1999). A Fas-dependent component in 5-
10 fluorouracil/leucovorin-induced cytotoxicity in
11 colon carcinoma cells. *Clin Cancer Res* 5, 425-430.
- 12 1. Nagata, S. Fas ligand-induced apoptosis. *Annu*
13 *Rev Genet*, 33: 29-55, 1999.
- 14 2. Griffith, T. S. and Lynch, D. H. TRAIL: a
15 molecule with multiple receptors and control
16 mechanisms. *Curr Opin Immunol*, 10: 559-563, 1998.
- 17 6. Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W.,
18 and Park, J. H. Increased expression of cFLIP(L) in
19 colonic adenocarcinoma. *J Pathol*, 194: 15-19, 2001.
- 20 7. Park, S. J., Kim, Y. Y., Ju, J. W., Han, B. G.,
21 Park, S. I., and Park, B. J. Alternative splicing
22 variants of c-FLIP transduce the differential signal
23 through the Raf or TRAF2 in TNF-induced cell
24 proliferation. *Biochem Biophys Res Commun*, 289:
25 1205-1210, 2001.
- 26 14. Chou, T. C. and Talalay, P. Quantitative
27 analysis of dose-effect relationships: the combined
28 effects of multiple drugs or enzyme inhibitors. *Adv*
29 *Enzyme Regul*, 22: 27-55, 1984.
- 30 15. Boyer, J., McLean, E. G., Aroori, S., Wilson,
31 P., McCulla, A., Carey, P. D., Longley, D. B., and
32 Johnston, P. G. Characterization of p53 wild-type

- 1 and null isogenic colorectal cancer cell lines
2 resistant to 5-fluorouracil, oxaliplatin and
3 irinotecan. Clin Cancer Res, 10, 2004.
- 4 16. Bannerman, D. D., Tupper, J. C., Ricketts, W.
5 A., Bennett, C. F., Winn, R. K., and Harlan, J. M. A
6 constitutive cytoprotective pathway protects
7 endothelial cells from lipopolysaccharide-induced
8 apoptosis. J Biol Chem, 276: 14924-14932, 2001.
- 9 39. Longley, D. B., Allen, W. L., McDermott, U.,
10 Wilson, T. R., Latif, T., Boyer, J., Lynch, M., and
11 Johnston, P. G. The roles of thymidylate synthase
12 and p53 in regulating Fas-mediated apoptosis in
13 response to antimetabolites. Clin Cancer Res, 10:
14 3562-3571, 2004.
- 15 40. Grambihler, A., Higuchi, H., Bronk, S. F., and
16 Gores, G. J. cFLIP-L inhibits p38 MAPK activation:
17 an additional anti-apoptotic mechanism in bile acid-
18 mediated apoptosis. J Biol Chem, 278: 26831-26837,
19 2003.
- 20 41. Levine, A. J., Momand, J., and Finlay, C. A.
21 The p53 tumour suppressor gene. Nature, 351: 453-
22 456, 1991.
- 23 42. Bartke, T., Siegmund, D., Peters, N.,
24 Reichwein, M., Henkler, F., Scheurich, P., and
25 Wajant, H. p53 upregulates cFLIP, inhibits
26 transcription of NF-kappaB-regulated genes and
27 induces caspase-8-independent cell death in DLD-1
28 cells. Oncogene, 20: 571-580, 2001.
- 29 43. Fukazawa, T., Fujiwara, T., Uno, F., Teraishi,
30 F., Kadowaki, Y., Itoshima, T., Takata, Y., Kagawa,
31 S., Roth, J. A., Tschopp, J., and Tanaka, N.
32 Accelerated degradation of cellular FLIP protein

1 through the ubiquitin-proteasome pathway in p53-
2 mediated apoptosis of human cancer cells. *Oncogene*,
3 20: 5225-5231, 2001.

4 44. Andre, T., Boni, C., Mounedji-Boudiaf, L.,
5 Navarro, M., Tabernero, J., Hickish, T., Topham, C.,
6 Zaninelli, M., Clingan, P., Bridgewater, J., Tabah-
7 Fisch, I., and de Gramont, A. Oxaliplatin,
8 fluorouracil, and leucovorin as adjuvant treatment
9 for colon cancer. *N Engl J Med*, 350: 2343-2351,
10 2004.

11 45. Zhou, X. D., Yu, J. P., Liu, J., Luo, H. S.,
12 Chen, H. X., and Yu, H. G. Overexpression of
13 cellular FLICE-inhibitory protein (FLIP) in gastric
14 adenocarcinoma. *Clin Sci (Lond)*, 106: 397-405, 2004.

15
16
17
18